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THE ROLE OF ARACHIDONIC ACID AND ITS
METABOLITES IN CONTROLLING INOSITOL
LIPID METABOLISM IN SWISS 3T3 CELLS

A thesis submitted to the University of Glasgow in
candidature for the degree of Doctor of Philosophy
in the Faculty of Science.

by

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Abbreviations

E.D.T.A.	-	Ethylenediamine tetraacetic acid
A.A.	-	Arachidonic acid
T.C.A.	-	Trichloroacetic acid
PtdA(PA)	-	Phosphatidic acid
PtdS	-	Phosphatidylserine
PtdCho	-	Phosphatidylcholine
PtdE	-	Phosphatidylethanolamine
PtdIns	-	Phosphatidylinositol
PtdInsP	-	Phosphatidylinositol-4-phosphate
PtdInsP ₂	-	Phosphatidylinositol-4,5-bisphosphate
InsP	-	Inositol monophosphate (for specific isomers see text)
InsP ₂	-	Inositol bisphosphate (for specific isomers, see text)
InsP ₃	-	Inositol trisphosphate (for specific isomers, see text)
CDP-DG	-	Cytidine diphosphate diglyceride
CTP	-	Cytidine triphosphate
cAMP	-	Cyclic-adenosine monophosphate
cGMP	-	Cyclic-guanosine monophosphate
ATP	-	Adenosine triphosphate
GTP	-	Guanosine triphosphate
PGF _{2α}) PGE ₂)	-	Prostaglandin F _{2α} ; E ₂
PGI ₂	-	Prostacyclin
c.o.	-	Cyclooxygenase
l.o.	-	Lipoxygenase
Hepes	-	N-2-hydroxyethylpiperazine-N'-Z-ethanesulphonic acid

BSS	-	Balanced salt solution
EGF	-	Epidermal growth factor
PDGF	-	Platelet derived growth factor
FDGF	-	Fibroblast derived growth factor
IGFI) IGFII)	-	Insulin-like growth factor I and II
PLA ₂	-	Phospholipase A ₂
PLC	-	Phospholipase C
DG	-	Diacylglycerol
T.P.A.	-	12-O-tetradecanoyl phorbol-13-acetate
P.M.A.	-	Phorbol 12-myristate 13-acetate

SUMMARY

Swiss 3T3 fibroblasts were chosen as a model system to study the role of arachidonic acid and/or its metabolites in controlling serum- and PDGF-induced inositol lipid metabolism. $\text{PGF}_{2\alpha}$ (0.01 - 10 μM) caused a rapid stimulation of inositol phospholipid hydrolysis which was maximal within 1-2 minutes of stimulation with the agonist. The level of PtdIns was not significantly affected by $\text{PGF}_{2\alpha}$. The time course of [^3H] - InsP accumulation suggests that InsP was not produced directly from PtdIns, but was formed by the dephosphorylation of InsP_2 and InsP_3 at least within 1 minute after the $\text{PGF}_{2\alpha}$ addition. 1.6 μM $\text{PGF}_{2\alpha}$ caused a two-three fold increase in [^3H] -PtdA levels, but did not affect the levels of any other major phospholipid measured.

Having shown that $\text{PGF}_{2\alpha}$ stimulated inositol lipid metabolism in 3T3 cells, it was postulated that the formation of prostaglandins from AA might be intermediary in the action of PDGF on inositol phospholipid hydrolysis. In order to prove this, it was necessary to be able to inhibit their formation. Surprisingly, the cyclooxygenase inhibitors tested, (flurbiprofen and indomethacin), on their own caused a stimulation of inositol lipid metabolism. This was observed as an increase in [^3H] -InsP levels and in [^3H] -PtdA levels. The inhibitors had no effect on the levels of any other major phospholipid. The effect of flurbiprofen on inositol lipid metabolism was shown to be dose-related, with 100 μM flurbiprofen producing the maximum stimulation.

By inhibiting cyclooxygenase activity, it seemed possible that flurbiprofen might have re-directed free AA towards the lipooxygenase pathway and thus the possibility that a stimulatory lipooxygenase metabolite was responsible for the increase in inositol phospholipid hydrolysis was investigated. BW755C, an inhibitor of both cyclooxygenase and lipooxygenase also caused a stimulation of inositol lipid metabolism, in a dose-related manner. The effect was maximal with 100 μ M BW755C. Although it remains possible that BW755C did not inhibit all the l.o. enzymes, it seemed more likely that the stimulation of inositol phospholipid hydrolysis was due to AA itself. Further evidence which suggested this to be the case was that flurbiprofen and BW755C, in a dose-dependent manner, caused an increase in the level of free [3 H] -AA in the medium surrounding the cells. The drugs also caused an increase in the level of [3 H] -arachidonyl PtdA.

Exogenously applied AA increased the level of [3 H] -InsP in a dose-related manner which was maximal with 100 μ M AA. This concentration of AA was thought too high to be of physiological significance. However, one contributing factor here was that the majority of the exogenously applied AA bound to albumin in the BSS surrounding the cells. In albumin-free BSS 0.1 μ M AA gave almost an equivalent increase in InsP formation as 100 μ M AA in cells surrounded by albumin-containing BSS. Also, in experiments where the effects of BW755C and indomethacin on [3 H] -AA release and on [3 H] -arachidonyl PtdA formation were studied, the results were

consistent with the idea that endogenously released AA was binding to albumin in the BSS surrounding the cells.

Further support for the theory that AA itself stimulated inositol lipid metabolism in 3T3 cells was that flurbiprofen and BW755C were not additive with exogenously applied AA in stimulating inositol phospholipid hydrolysis. This suggests that they have the same pathway of action.

Studies were then carried out to investigate the pathway by which AA was liberated. Melittin, a PLA_2 activator, stimulated both AA release and inositol lipid metabolism in a dose-related manner. Dexamethasone, an indirect inhibitor of PLA_2 , abolished the BW755C- and flurbiprofen-induced increase in inositol lipid metabolism in a dose-related manner. These results suggest that the PLA_2 pathway is involved in the liberation of AA from the membrane phospholipids in 3T3 cells. The possibility that the phospholipase C/diglyceride lipase pathway is also involved was not studied.

In an attempt to prove the involvement of AA in the PDGF-induced increase in inositol lipid metabolism, three strategies were devised:

- 1) An investigation of the effect of combining submaximal concentrations of dialysed serum with BW755C. Enhancement of the serum's effects by BW755C might suggest that they employed the same mechanism of action.
- 2) An investigation of the effects of pertussis toxin on AA release and inositol lipid metabolism. It had been suggested that pertussis toxin could selectively block agonist-induced

AA release leaving the increase in inositol lipid metabolism unaffected. Thus, if the pertussis toxin inhibited both AA release in inositol lipid metabolism in BW755C-stimulated 3T3 cells, then this would suggest that the increase in inositol lipid metabolism was dependent on the release of free AA.

3) An investigation of the effect of dexamethasone on PDGF-induced inositol lipid metabolism. If dexamethasone were to abolish or partially inhibit the PDGF-induced increase in inositol lipid metabolism, this might suggest that the liberation of AA via PLA_2 was involved.

The results of these studies were inconclusive. Synergism between BW755C and dialysed serum was observed with certain concentrations of the drugs. However, the results observed for other concentrations of drugs suggested the occurrence of additivity and even inhibition. Pertussis toxin had no effect on agonist-stimulated [3H] -AA release or on [3H] -arachidonyl PtdA formation. Finally, dexamethasone was observed to partially inhibit the PDGF-induced increase in inositol lipid metabolism. However, further study is necessary to support this finding. Also the effect of PDGF on inositol lipid metabolism was only inhibited by about 20% and this might suggest that the involvement of AA in the PDGF-induced turnover of inositol lipid metabolism might be only minor.

1. INTRODUCTION

1. Introduction

In a multicellular organism, the individual cells exist in three states, with respect to proliferation. Post-mitotic cells, such as red blood cells, fused muscle cells and the bulk of the central nervous system neurons, are terminally differentiated and incapable of cell division. Actively-dividing cells, such as those of the intestinal crypt villi, are, as their name suggests, continually dividing. Quiescent or non-proliferating cells are cells which have the potential to re-enter the cell cycle and divide in response to an appropriate mitogenic signal. The stimulus for recruitment of this latter class of cells to divide is dependent on cell type. Hepatocytes respond to surgical resection, antibody producing cells to foreign antigen, mammary cells to elevated circulating levels of insulin and prolactin, and fibroblasts respond to wounding. Appropriate initiators, whether exogenous molecules, endogenous hormones and growth factors or physical injury, evoke a cell-type specific response in the non-proliferating cells which compels them to enter the division cycle. The regulation of growth control, that is, the decision to enter or exit the cycle in response to environmental cues, is an intricate and complex phenomenon for which much of the molecular basis is poorly understood (Herschman et al., 1982).

1.1 The control of growth of mammalian cells

In the whole animal, growth of cells is under three types of control. These can be exerted either alone, or

in combination with each other.

Endocrine control of growth is due to the release of hormones from endocrine glands such as the pituitary gland. These hormones e.g. growth hormone and melanocyte-stimulating hormone, are dispersed into the blood stream, which carries them to their site of action. These hormones can act on cellular receptors some distance from the endocrine gland from which they were secreted, to cause a characteristic change in the cell's growth.

Paracrine control is exerted by one cell on a neighbouring cell by the release of growth-affecting molecules or ions. For example, in response to wounding, growth factors, such as platelet derived growth factor, are released from blood platelets. The growth factors act on the receptors of neighbouring epithelial cells and fibroblasts to stimulate proliferation of these cells.

Autocrine control is the control of growth exerted by a cell on itself, either by releasing growth-influencing factors outside the cell, which in turn act on the cell's own receptors on the external surface of the cell's membrane, or by releasing these factors internally into the cytosol to react with receptors in the nucleus or on internal cellular membranes. An example of this is when a small T-lymphocyte is activated by a specific antigen, it divides and proliferates to produce a clone. These cells are known as "killer cells" and their combination with antigen results in the release of a number of factors including a substance that induces lymphocyte division (mitogenic factor). This

mitogenic factor can act on neighbouring lymphocytes (paracrine control) and also on the lymphocyte itself in an autocrine fashion (Katz, 1977).

All of these growth controlling mechanisms can be disrupted by neoplasia of appropriate cells. For example, oversecretion of growth hormone is often caused by an acidophil tumour of the pituitary, and this leads to a condition known as acromegaly, which is characterised by enlargement of the hands and feet and protrusion of the lower jaw.

In their search for a rational understanding of the processes which lead to neoplasia, scientists have examined cell growth in vitro, using cells grown in culture.

It is widely observed that normal, untransformed cells show "density-dependent regulation", that is, they tend to grow to a certain saturation density and then stop growing. The resulting quiescent cells can remain both healthy and quiescent for some time. In contrast, "transformed" cells have lost the process of density-dependent regulation and will grow continuously until the supply of nutrients in the medium has been exhausted. These cells will not become quiescent and must be transferred to fresh medium or they will die (Holley, 1975).

Density-dependent regulation was initially thought to be due to "contact inhibition". It was postulated that specific surface receptors are activated by cell-cell contact and generate a negative signal which halts further growth (Rozengurt, 1980). Although the term "contact inhibited" is still used in the literature to describe quiescent cells, many lines of evidence indicate that quiescence is not caused

by cell-cell contact. Instead, arrest of growth of mammalian cells is explained by the "humoral hypothesis". This proposes that high cell density limits the availability of medium components, particularly growth factors present in serum (Holley, 1975).

In support of this theory, it has been observed that the growth of Swiss 3T3 cells, a mouse embryonic cell line, is normally controlled by macromolecules in serum (Holley and Kiernan, 1968). The saturation density is directly proportional to the initial serum concentration in the medium. However, if a growth factor like PDGF is added further growth will occur. Thus, the growth of normal cells is probably controlled by interactions of the cells with a variety of polypeptide hormones or hormone-like growth factors present in the surrounding medium. Transformed or malignant cells escape from normal growth controls by requiring less of the hormones or growth factors (Holley, 1975), and thus can initiate growth in response to lower external concentrations of growth factors. It is important to note that most transformed cells retain some dependence upon growth regulatory factors of exogenous or endogenous origin and thus manipulation and production or action of growth factors has a role to play in the treatment of proliferative disorder.

1.1.1 Growth promoting molecules

As discussed above, under usual cell culture conditions the limiting component for proliferation is the concentration of serum present in the medium (Holley, 1975). The addition of serum to quiescent cultures enhances the rates of protein and RNA synthesis and dramatically stimulates DNA synthesis and cell division. A molecular approach to the study of growth regulation requires the use of defined external stimuli (mitogens). Thus, pure molecules such as outlined in Table 1 constitute the necessary tools with which to explore the nature of the surface receptors related to growth control.

1.1.2 The use of Swiss 3T3 cells in studying proliferation

Swiss 3T3 cells originated from cultures of 6-8 day old Swiss Mouse embryos (Todaro and Green, 1963). They are a good model system for studying proliferation as they respond to a vast number of chemically diverse molecules which can stimulate quiescent cells (in the G_0 state) to enter the proliferating pool (G_1). The receptor types for these growth-promoting molecules can be split into three broad groups on the basis of their proposed transduction pathways. It is important to point out here, that the action of some of these growth-promoting molecules mentioned (Table 1) may not be limited to one of these pathways.

Table 1: Receptors present on Swiss 3T3 cells

Receptor type	Transducing mechanism	Fully Mitogenic?	Co-mitogenic with insulin?
PDGF	Tyrosine Kinase / Ptd.Ins / cAMP (↑)	Yes	Yes
EGF		No	Yes
Insulin		No	-
PGF _{2α}	PtdIns	No	Yes
Bombesin	PtdIns/cAMP (↑)	Yes	Yes
Vasopressin	PtdIns	No	Yes
PAF	PtdIns	No	?
Adenosine	cAMP (↑)	No	Yes
PGE ₁ /PGI ₂	cAMP (↑)	No	Yes

The table shows some of the receptors present on Swiss 3T3 cells, and their proposed signal transduction pathways. The term "fully mitogenic" refers to the ability of a growth factor, acting on its receptor, to act early in the transition from the quiescent state (G₀) to G₁ and to facilitate the subsequent actions of other growth factors. It does not imply that certain growth factors, acting on their receptors, are ineffective in causing mitosis (proliferation) of 3T3 cells. "Co-mitogenic with insulin" refers to the ability of growth factors, acting on their receptors, to synergise with insulin in stimulating DNA synthesis and thus proliferation of 3T3 cells.

1.1.3 Tyrosine kinase

Protein kinases transfer the (terminal) γ -phosphate of the cellular energy carrier adenosine triphosphate (ATP) to an appropriate amino acid residue in a protein and thus alter the action of that protein.

Most protein kinases phosphorylate proteins on either serine or threonine residues. Certain growth factor receptors (see later in this section) and enzymes encoded by oncogenes (ref. Section 1.2) such as "pp60src" were found instead to catalyze the transfer of phosphate from ATP to tyrosine (Cooper and Hunter, 1983).

Tyrosine phosphorylation has been implicated in the complex regulatory systems that maintain cellular shape and control of growth of cells, but its role and mechanism of action are still largely unknown.

1.1.3.1 Tyrosine kinase-linked receptors

To understand how protein-tyrosine kinases regulate cell growth necessitates the identification of their substrates. This quest has involved various approaches including, for example, electrophoresis to analyse phosphoproteins found in normal versus transformed or growth factor-stimulated cells (Cooper et al., 1982), and the development of both poly- and monoclonal antibodies directed towards phosphotyrosine or analogues thereof, (Ek and Heldin, 1984 ;Friedman et al., 1984).

If the common enzymatic activity of tyrosine phosphorylation reflects a common mechanism of cell transformation, one might

predict that certain proteins should be universal targets. The murine 39 kilo-dalton (kDa) protein appears to be homologous to a phosphotyrosyl protein in transformed chick embryo fibroblasts, suggesting it could represent a key target. This protein, termed p36, has a molecular mass variously reported to be 34-39 kDa (Radke and Martin, 1979). The transforming protein of Rous sarcoma virus, pp60^{v-src}, can directly phosphorylate p36 in vitro, most likely at the same site that is found in vivo (Erikson et al., 1980). By cell fractionation and immunofluorescence studies, p36 appears to be a peripheral membrane protein, located on the cytoplasmic face of the plasma membrane (Nigg et al., 1983; Radke et al., 1983).

The complementary DNA (cDNA) sequences of rat lipocortin, a phospholipase A₂ inhibitory protein (see Section 1.1.6), and murine p36 have been determined and show an overall homology of 50% at the deduced amino acid level (Geisow et al., 1986). This led to the proposal that tyrosine kinase-linked growth factors may act through arachidonic acid (AA) metabolism (see Sections 1.1.6 and 1.3).

Another candidate as a protein-tyrosine kinase substrate is "enolase", which is involved in the glycolytic pathway in cells. Cooper et al. (1983b) demonstrated by immunoprecipitation, that enolase became phosphorylated on tyrosine following transformation by a variety of retroviruses.

Eigenbrodt et al. (1983) purified enolase from transformed chick embryo fibroblasts and showed it to be a 41 kDa protein.

Finally, two other phosphotyrosyl-proteins have been investigated in some detail in transformed cells: a protein of 50 kDa (termed pp50) and pp60^{v-src} itself. In cells transformed by Rous sarcoma virus (RSV), the pp50 protein is found in a complex with pp60^{v-src} and a third protein pp90 (Oppermann et al., 1981a; Eigenbrodt et al., 1983). Both pp90 and pp50 are cellular proteins unrelated structurally to pp60^{v-src}. The function of pp50 in normal cells remains to be established. The protein, pp90, appears to be a "stress" protein, whose synthesis is induced by either heat shock or glucose deprivation (Oppermann et al., 1981b; Lanks et al., 1982). The pp50-pp60^{v-src}-pp90 complex is cytoplasmic and may be involved in the transport of newly synthesized pp60^{v-src} to the membrane (Courtneidge et al., 1982; Brugge et al., 1983).

1.1.3.1.1 Epidermal growth factor

Epidermal growth factor (EGF) is a polypeptide isolated from the submaxillary glands of the male mouse (Savage et al., 1972), which exhibits growth-stimulating activity on various epidermal and epithelial tissues, both in vivo and in vitro. Amino acid analysis revealed that mouse EGF is a 53-residue polypeptide (Taylor et al., 1972). The primary sequence of mouse EGF and the position of the three internal disulphide bonds have been determined (Savage et al., 1972; Savage et al., 1973).

Human EGF has been purified from urine and urogastrone, a gastric antisecretory polypeptide also purified from urine

(Gregory , 1975), which is probably identical to human EGF, has been sequenced. It is also a 53-residue polypeptide with a very high degree of homology to mouse EGF.

A wide variety of in vivo and in vitro responses to EGF have been documented (Carpenter and Cohen, 1979). For responsive cells in culture, certain biochemical changes can occur within seconds or minutes of the addition of EGF to the medium, while others require hours. Some or all of these responses might be involved in the pathway leading to mitosis. A431 cells are useful models for studying the action of EGF. These cells, derived from a human epidermoid carcinoma (Girard et al., 1983) have an abnormally high number of plasma membrane receptors for EGF (Haigler et al., 1978). EGF is not mitogenic for this cell line, but rather is inhibitory to the growth of A431 cells (Gill and Lazar, 1981). In contrast, EGF stimulates DNA synthesis in many other cell types including Swiss 3T3 fibroblasts (Carpenter and Cohen, 1979).

The 170 kDa receptor protein for EGF has been identified and purified by Cohen et al. (1982). Experiments by Carpenter et al. (1975) and Carpenter and Cohen (1976), suggested that, after binding to the receptor, the EGF-receptor complex is clustered in "coated pits" which invaginate to form endocytotic vesicles (i.e. the complex is internalized). These vesicles merge with primary lysosomes which fuse with secondary lysosomes in which the hormone and, most probably, the receptor are degraded.

Within the single polypeptide chain of the EGF receptor, there is a domain which functions as an EGF binding site and as an EGF-stimulable tyrosyl-specific protein kinase. This protein is disposed as a transmembrane allosteric enzyme in the plasma membranes of target cells, such that the effector site (i.e. the EGF binding site) is on the outside of the cells and the catalytic site is accessible to the cytoplasm (Yarden et al., 1986 and Figure 1). The primary transmembrane signalling event is thought to be the activation of the kinase which results from binding of EGF to the receptor site.

The cell surface receptors for EGF and insulin (see Section 1.1.3.1.4) appear to share a common evolutionary origin as suggested by structural similarity of cystein-rich regions in their extracellular domains and a highly conserved tyrosine-specific protein kinase domain (Riedel et al., 1986). Only minor similarity is found outside this catalytic domain, as expected for receptors that have different ligand specificities and generate different biological signals. Studies using a chimaeric receptor molecule comprising the extracellular portion of the insulin receptor joined to the transmembrane and intracellular domains of the EGF receptor, have shown that the EGF receptor kinase domain of the chimaeric protein, is activated by insulin binding (Riedel et al., 1986). This suggests that insulin and EGF receptors employ closely related or identical mechanisms for signal transduction across the plasma membrane.

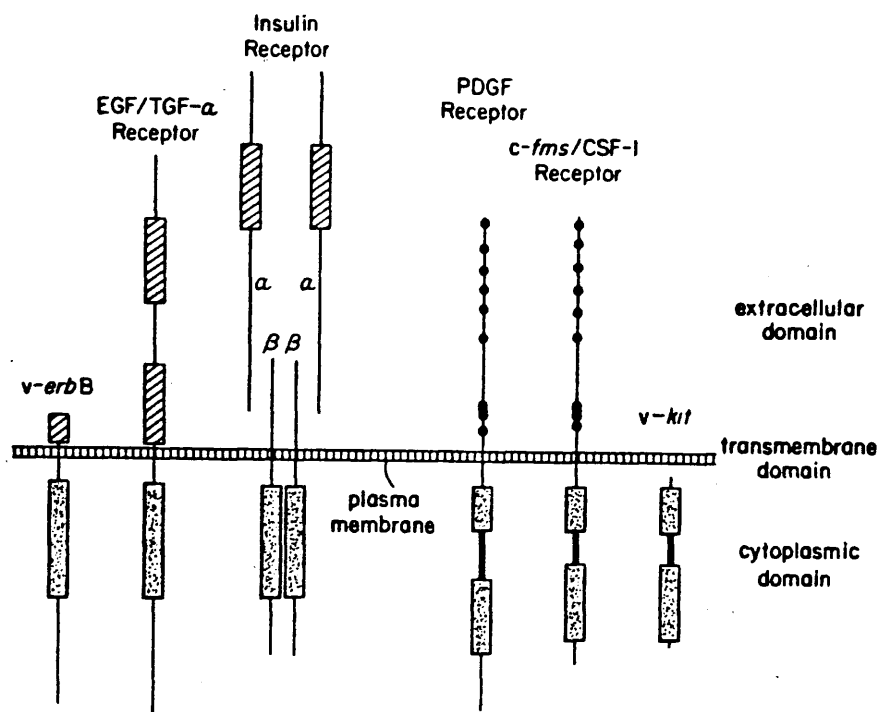


Figure 1: Topological comparison of the two groups of tyrosine-kinase receptors and their oncogenic variants

All proteins shown are oriented so that their carboxy-termini are in the cytoplasm. Hatched boxes indicate regions that are rich in cysteine residues. Protein domains that share high homology with other tyrosine-kinases of the src gene family are shown by stippled boxes. Solid lines show stretches of sequences inserted within the tyrosine-kinase regions of PDGF receptor, c-fms, and v-kit. The distribution of cysteine residues in the extracellular domains of PDGF-receptor and c-fms/CSF-1 receptor is indicated by closed circles, whereas hatched boxes show cystein-rich repeat domains found in the extracellular ligand-binding regions of EGF receptor and insulin receptor. Taken from Yarden et al. (1986).

The transforming protein of RSV (pp60^{v-src}) is a protein kinase specific for tyrosyl residues, as are the gene products of a number of other transforming viruses (Hunter and Setton, 1980; see also Section 1.2). Also, other mitogenic or co-mitogenic polypeptides including PDGF and insulin have been demonstrated to stimulate receptor-associated tyrosyl protein kinases. Thus, the regulation of tyrosyl kinase activity is thought to play an important role in growth regulation.

Since the EGF-stimulable kinase, like pp60^{src}, was found to be specific for tyrosyl residues (Ushiro and Cohen, 1981), considerable attention has been focussed on the possible relationship between the EGF receptor/kinase and the products of various oncogenes (see Section 1.2). Interest was greatly heightened when it was found that there is a close sequence homology between the EGF receptor/kinase and one of the two transforming gene products of avian erythroblastosis virus, "v-erb" (Downward et al., 1984).

Addition of tumour promoters, such as 12-O-tetradecanoyl phorbol-13-acetate (TPA) to cells, results in a number of alterations in the EGF receptor. Studies in a variety of cell types, including both mouse and human epidermal and fibroblast lines, have shown that the addition of TPA induces the loss of EGF binding (Lee and Weinstein, 1978; Shoyab et al., 1979). The majority of studies indicate that this decrease in EGF binding results from a decrease in affinity rather than receptor number (Friedman et al.,

1984). It has been suggested that protein kinase C (thought to be activated by TPA) may act as a normal cellular regulator of EGF (Hunter et al., 1984). Addition of EGF to certain cell types (e.g. A⁴31 cells) can induce a small transient turnover of phosphatidylinositol and other inositol-containing lipids, resulting in the production of diacylglycerol and the internal release of calcium ions (see Section 1.1.4 and Habenicht et al., 1981). This may result in the activation of protein kinase C, thereby acting as a feedback inhibitory mechanism for desensitization of the EGF receptor (Friedman et al., 1984).

1.1.3.1.2 Platelet-derived growth factor

Platelet-derived growth factor (PDGF) is a polypeptide that, in particular, stimulates the proliferation of connective tissue cells. It has been purified to homogeneity by Antoniades et al. (1979) and Heldin et al. (1979), as a heat stable (100°C), cationic protein composed of two 30 kDa disulphide-linked chains, denoted A and B (Johnsson et al., 1982).

Evidence based on both in vivo and in vitro studies indicates that the binding of PDGF to its cell surface receptor leads to the activation of a receptor-associated protein-tyrosine kinase (Ek et al., 1982; Ek and Heldin, 1984 and Figure 1). Incubation of plasma membranes from human fibroblasts, Swiss 3T3 cells or glial cells with ³²P-ATP, for example, revealed a protein-tyrosine kinase activity which could be stimulated by PDGF (Ek et al., 1982; Nishimura et al., 1982). The major endogenous phosphate-acceptor was

a protein of 170-180 kDa, which appears to correspond to the PDGF receptor itself (Heldin et al., 1983). Similar results have also been obtained by in vivo studies of ^{32}P -labelled fibroblasts stimulated with PDGF and subjected either to gel electrophoretic analysis or immunoprecipitation with an anti-phosphotyrosine antibody (Ek et al., 1984).

Thus, PDGF is similar to EGF in stimulating tyrosine kinase activity. Like EGF it also stimulates a number of growth associated responses such as cytoskeletal rearrangement (Mellstrom et al., 1983) and enhanced expression of a family of genes including the c-myc (Kelly et al., 1983) and c-fos (Greenberg and Ziff, 1983) oncogenes (see Section 1.2). However unlike EGF, PDGF can stimulate hydrolysis of phosphatidylinositol 4,5-bisphosphate (PtdInsP_2) in 3T3 cells (Berridge et al., 1984a; Besterman et al., 1986). (EGF has been shown to stimulate PtdInsP_2 hydrolysis in A431 cells).

Similarly to EGF, after binding, PDGF is rapidly internalized and degraded in the lysosomes (Rozengurt, 1980). Also, PDGF has been shown to generate a transient (less than 1 hour) production of diacylglycerol (DG) (Habenicht et al., 1981), and the conversion of inactive cytoplasmic protein kinase C to an active, membrane-associated state. Thus, a further consequence of PDGF action is the enhanced phosphorylation of the EGF receptor on serine and threonine residues (Rosner et al., 1985) and a reduction of EGF binding to the population of high affinity receptors (Rozengurt et al., 1983a; Brown et al., 1984).

1.1.3.1.3 Fibroblast-derived growth factor

Fibroblast-derived growth factor (FDGF) is a polypeptide of apparent molecular weight 24 kDa. It can be isolated from the conditioned medium of SV-40 (simian virus)-transformed BHK cells (Bürk, 1976; Dicker et al., 1981). Rozengurt (1980) noted that FDGF stimulates DNA synthesis in 3T3 cells. This growth factor has certain PDGF-like characteristics and Dicker et al. (1981) suggested that FDGF and PDGF belonged to the same family of growth factors. Stroobant et al. (1985) purified a FDGF-like molecule from SV-28 conditioned medium and reported it to be a hydrophobic and cationic protein of apparent molecular weight 31 kDa, containing disulphide-linked polypeptides. These properties are shared by PDGF. Also, FDGF is immunologically related to PDGF, as human PDGF antiserum immunoprecipitates with purified FDGF (Stroobant et al., 1985). Further evidence linking these two growth factors is that FDGF is a potent mitogen for Swiss 3T3 cells comparable in potency to PDGF (Dicker et al., 1981).

1.1.3.1.4 Insulin and insulin-like growth factors

Insulin

Insulin is a small polypeptide of M.W. 5700. It has been isolated from the β -cells of the Islets of Langerhans in the endocrine pancreas, and is primarily responsible for the stimulated uptake of glucose by muscle and adipose tissue in response to elevated blood glucose from the portal vein of the liver. It is composed of two peptide chains united

by disulphide bonds. The A chain has 21 and the B chain 30 amino acid residues. As a major hormonal regulator of certain metabolic processes, it interacts with growth hormone, glucagon, glucocorticoids, adrenaline, and noradrenaline to promote nutrient storage, synthetic processes and to regulate the energy supply to tissues (Houslay, 1985).

Insulin acts at its receptor in the plasma membrane and like the PDGF and EGF receptors, it becomes phosphorylated at tyrosine in insulin-treated hepatoma cells (Kasuga et al., 1982a,b and see Figure 1). Various studies have demonstrated that the insulin binding site lies on a 135 kDa protein subunit of the receptor (Houslay, 1985). This has been termed the α -subunit of the insulin receptor. A small amount of labelling has also been noted on a component of about 90 kDa. This was subsequently shown to be the β -subunit of the receptor (Massagué et al., 1980; Czech, 1981).

Studies on the insulin receptor have revealed that both TPA and β -adrenergic agonists modulate insulin action. Enhanced phosphorylation of the β -subunit of the insulin receptor on phosphoserine residues following treatment with TPA have been reported. In hepatoma cells, TPA appears to have no effect on binding despite a 2-fold reduction in the ability of insulin to induce phosphorylation of its receptor and to activate the enzymes glycogen synthase and tyrosine amino transferase (Takayama et al., 1984).

As in the case of EGF, β -adrenergic agents also decrease the binding of insulin in rat adipocytes (Pessin et al., 1983) and numerous studies have documented antagonism between insulin and elevated cAMP levels (e.g. Foulkes et al., 1982).

Insulin-like growth factors

In blood only a small proportion of insulin-like activity is represented by insulin (Zapf et al., 1981). Antibodies specific for insulin, when added to serum, blocked the effects of insulin, but did not block the major "insulin activity". This activity was termed "non-suppressible insulin-like activity" or NSILA (Froesch et al., 1963). NSILA consists of at least three substances: a large molecular weight protein, NSILA-P (Poffenbarger, 1968), and two small molecular weight polypeptides that have been purified to homogeneity and whose chemical structures have been elucidated. These two polypeptides have been termed "insulin-like growth factors (IGF) I and II (Rinderknecht and Humbel, 1978a, b). There is a striking similarity between proinsulin, IGF I and IGF II, suggesting that the three polypeptides are homologs (Zapf et al., 1981).

The IGF I receptor is structurally similar to the insulin receptor and is composed of α and β subunits $M_r = 130$ kDa and 98 kDa respectively. The IGF II receptor is a monomeric structure which has an apparent $M_r = 220-250$ kDa (Kasuga et al., 1981; Jacobs et al., 1983). The primary structure of the human IGF II receptor, predicted from the

cDNA sequence, reveals a transmembrane receptor molecule with a large extracellular domain, made up of fifteen repeat sequences and a small region homologous to the collagen-binding domain of fibronectin. The structural and biochemical features of the IGF II receptor appear identical to those of the cation-independent mannose-6-phosphate receptors (Morgan et al., 1987).

IGF I is generally accepted to be a highly potent mitogen acting through its own type I IGF receptors. Although IGF II has been shown to stimulate DNA synthesis and cell replication in a number of cell types (Houslay, 1985), cross-reactivity of IGF II with IGF I and insulin receptors has complicated interpretation of these studies in terms of the function of the type II IGF receptor. IGF I, IGF II and insulin all interact to some degree with the others' receptors, with the exception that insulin does not appreciably bind to IGF II receptors (Massagué and Czech, 1982).

IGF I receptors have been shown to have an associated tyrosine kinase activity (Jacobs et al., 1983; Rubin et al., 1983) but the transduction pathway for the type II receptor has yet to be established. In 3T3 cells, insulin alone does not have appreciable mitogenic activity, but it can markedly potentiate the effect of mitogens e.g. EGF (Jimenez de Asua et al., 1981). Another unresolved question is whether 3T3 cells contain receptors for insulin or for IGF I.

1.1.4 Inositol lipid metabolism

Myo-inositol has long been known as an essential nutrient promoting animal growth (Kuksis and Mookerjee, 1978) and cell division (Eagle et al., 1957). The growth-promoting action of myo-inositol is believed to be mediated through its function as a substrate for the biosynthesis of three minor membrane lipids namely, phosphatidylinositol (PtdIns), phosphatidylinositol 4-phosphate (PtdIns(4)P) and phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P₂) (Figure 2). Inositol lipids have a characteristic fatty acid pattern; a high proportion of molecules contain a stearyl (C18:0) residue at position 1 and an arachidonyl (C20:4) residue at position 2 (Michell, 1975). Although the polyphosphoinositides are present in smaller amounts than PtdIns, it now appears that these lipids may play a central role in signal transmission (Berridge and Irvine, 1984).

The possible role of the phosphoinositides in cell function emerged from the experiments conducted by Hokin and Hokin (1953), who noted that incorporation of [³²P] - phosphate into phospholipids in pancreas (i.e. turnover of inositol phospholipids) was stimulated by acetylcholine, acting via a muscarinic receptor. It later became clear that a stimulated catabolism of inositol lipids occurs in many different cells in response to a wide variety of external signals (Michell, 1975; Downes and Michell, 1982; Berridge, 1984). Initially, PtdIns was regarded as the primary inositol-containing lipid that was degraded after

receptor occupancy. The term "phosphatidylinositol or PI response" was used to describe a sequence of events which was believed to be initiated by the breakdown of plasma membrane-located PtdIns (Figure 2) (Michell, 1975). However recent evidence suggests that the initial receptor-controlled reaction is the hydrolysis of PtdIns(4,5)P₂ by the action of phospholipase C (Berridge, 1984). PtdInsP₂ is located in the inner leaflet of the plasma membrane and is formed by a two-stage phosphorylation of PtdIns; firstly at the 4-position of its inositol head group by a specific kinase, to form PtdIns(4)P and secondly at the 5-position to give PtdIns(4,5)P₂ (Berridge, 1984 and Figure 2). As well as the two kinases responsible for the stepwise phosphorylation of PtdIns to PtdIns(4,5)P₂, there are corresponding phosphomonoesterases which convert PtdIns(4,5)P₂ back to PtdIns. These kinases and phosphomonoesterases constitute two linked phosphatidylinositol/PtdIns(4,5)P₂ futile cycles whereby phosphates are constantly being added to and removed from the 4 and 5 positions of the inositol head group. These futile cycles expend metabolic energy and this may explain why metabolic inhibitors are so effective in curtailing this signal transduction pathway (Berridge, 1984). Receptor occupation by an agonist transfers PtdInsP₂ out of the futile cycle by activating phospholipase C, which hydrolyses the lipid to form 1,2-diacylglycerol (DG) and inositol (1,4,5)-trisphosphate (Ins,(1,4,5)P₃) (Figure 2 and Berridge and Irvine, 1984). At first it was

considered possible that this reaction was a consequence of an increase in the free cytosolic calcium concentration (the calcium signal in stimulated tissue) rather than its cause. However in 1975 Michell noted that enhanced phosphoinositide metabolism could be experimentally dissociated from Ca^{2+} mobilization. Depletion of cellular Ca^{2+} with Ca^{2+} -deficient medium inhibited various cellular responses dependent on Ca^{2+} mobilization with little effect on phosphoinositide turnover. Further, when cytosolic Ca^{2+} was artificially increased using Ca^{2+} ionophores, a variety of Ca^{2+} -dependent cellular responses were activated, but phosphoinositide turnover was increased very little. From this and other approaches, he argued that the inositol lipid cycle might, in some way, serve as a biochemical coupling mechanism linking receptor activation to cellular Ca^{2+} mobilization.

It now seems that receptor-mediated Ca^{2+} changes occur in two phases (Putney, 1978; Putney et al., 1981), a release of Ca^{2+} to the cytosol from internal pools and a sequential or concomitant entry of Ca^{2+} into the cytosol from the extracellular space. The available evidence suggests that the internal pool from which Ca^{2+} is released is most likely a component of the endoplasmic reticulum (Burgess et al., 1983; Smigel et al., 1984). To couple receptor activation to internal Ca^{2+} release, it has been suggested that a second messenger is formed at the plasma membrane, which signals this release from the appropriate

intracellular pool. Berridge (1983) first suggested that the second messenger might be a product of polyphosphoinositide breakdown, specifically, inositol (1,4,5) trisphosphate ($\text{Ins}(1,4,5)\text{P}_3$). It has been shown for a number of different cell types, including Swiss 3T3 cells (Berridge et al., 1984a), that InsP_3 is rapidly formed in response to receptor activation and is capable of releasing Ca^{2+} from the endoplasmic reticulum (Berridge, 1983; Streb et al., 1983; Berridge and Irvine, 1984; Streb et al., 1984; Putney, 1986).

The rate at which $\text{Ins}(1,4,5)\text{P}_3$ is formed after cell stimulation, is fast enough for it to be the second messenger causing mobilization of Ca^{2+} (Berridge, 1983; Berridge and Irvine, 1984; Berridge et al., 1984b) and the concentration which it can reach in stimulated tissues may be in the micro-molar range which is within the levels active on permeabilized cells (Berridge and Irvine, 1984).

$\text{Ins}(1,4,5)\text{P}_3$ is dephosphorylated to inositol 1,4-bisphosphate ($\text{Ins}(1,4)\text{P}_2$) and then further dephosphorylated to either inositol-1-phosphate (Ins1-P) or inositol 4-phosphate (Ins4-P).

Recently, two naturally occurring isomers of InsP_3 have been identified: inositol (1,4,5) P_3 and inositol (1,3,4) P_3 (Irvine et al., 1984). Presently much research into the routes of formation and degradation of these isomers is being carried out. This has led to the discovery of inositol 1,3,4,5-tetrakisphosphate ($\text{Ins}(1,3,4,5)\text{P}_4$) (Batty et al.,

1985), which is formed from the phosphorylation of $\text{Ins}(1,4,5)\text{P}_3$ through a newly discovered 3-kinase (Irvine et al., 1986). $\text{Ins}(1,3,4,5)\text{P}_4$ was first identified in brain cortical slices (Batty et al., 1985) and appears to be the precursor of $\text{Ins}(1,3,4)\text{P}_3$ (Batty et al., 1985; Hawkins et al., 1986; Downes et al., 1986). When cells are stimulated by an agonist, not only do they produce $\text{Ins}(1,4,5)\text{P}_3$ but, after a short lag, they also begin to form $\text{Ins}(1,3,4)\text{P}_3$ (Irvine et al., 1986). When $\text{Ins}(1,4,5)\text{P}_3$ is produced by phospholipase C it can thus be metabolised by two separate routes, a degradative pathway to free inositol as outlined above or via a novel pathway to generate other inositol polyphosphates which could have additional messenger functions (Batty et al., 1985; Irvine et al., 1986) (Figure 2).

$\text{Ins}(1,3,4,5)\text{P}_4$ like $\text{Ins}(1,4,5)\text{P}_3$ is metabolized rapidly by a 5-phosphatase and this, and its rapid formation after receptor stimulation, has provoked debate on its potential as yet another inositol lipid-linked second messenger molecule (Downes, 1986). Very recently, the first evidence that this may be the case has been supplied by Irvine and Moor (1986), who suggest that $\text{Ins}(1,3,4,5)\text{P}_4$ may control some aspect of calcium influx across the plasma membrane, in particular that concerned with the refilling of the $\text{Ins}(1,4,5)\text{P}_3$ -sensitive calcium pool in the endoplasmic reticulum. Figure 2 outlines the steps which yield the inositol phosphate isomers found in mammalian cells, and reviewed in more detail by Downes (1986).

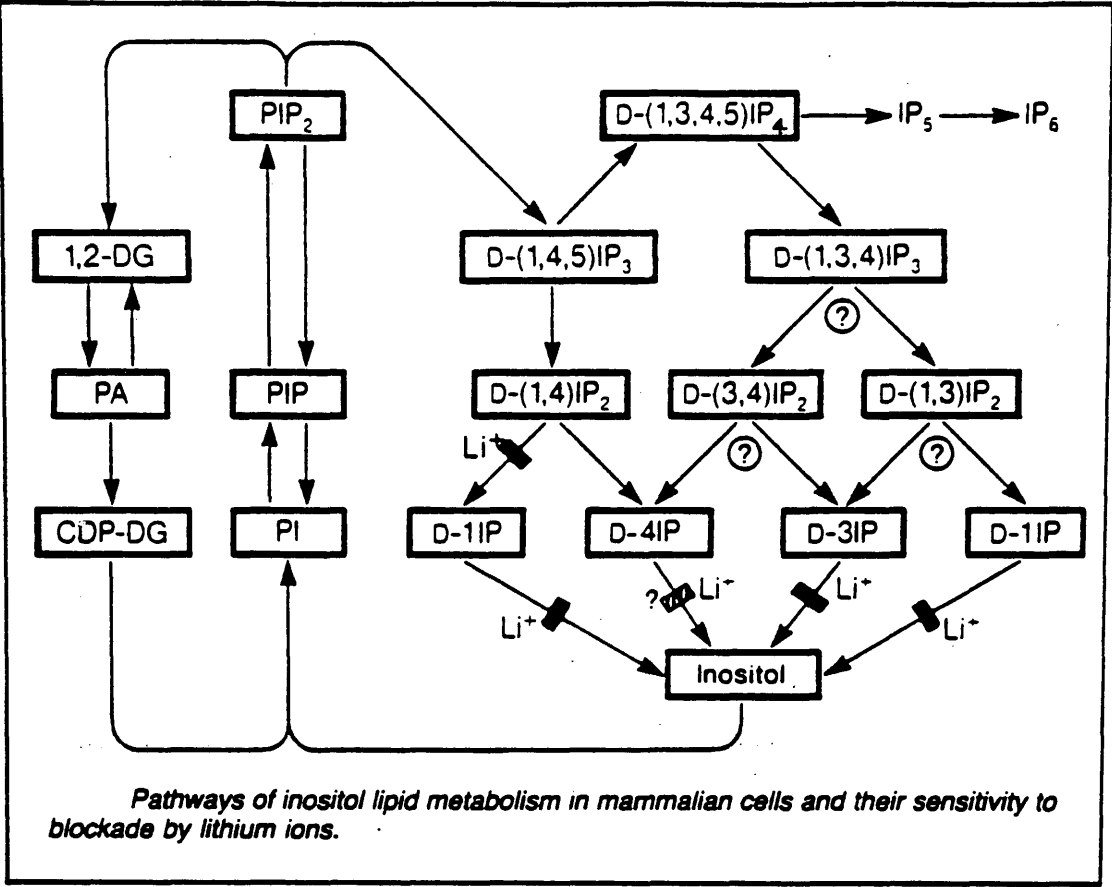


Figure 2: Pathways of inositol lipid metabolism in mammalian cells

Lithium and inositol lipid metabolism

Lithium is a useful pharmacological tool for studying the inositol lipid cycle as it is one of the few agents which interferes with the cycle in a selective manner. The brain of animals receiving lithium shows a large decrease in inositol and increase in total inositol monophosphate (Sherman et al., 1986). This is due to the blockade of the metabolic cascade at the monophosphate level; a feature which is evident in intact cells at extracellular concentrations above 0.1 mM (Drummond, 1987). The metabolic pathways thought to be blocked by lithium are shown in Figure 2. Thus, while the effects of lithium are clearly more complex than was originally surmised, the dominant effect of the ion to block inositol monophosphatase activity, allows it to amplify agonist-stimulated inositol lipid turnover.

Diacylglycerol and protein kinase C

The other product of $\text{PtdIns}(4,5)\text{P}_2$ hydrolysis by phospholipase C, is 1,2-diacylglycerol (DG). Phosphorylation of DG by DG-kinase results in the formation of phosphatidic acid (PtdA) (Figure 2). PtdA is subsequently conjugated with cytidine triphosphate (CTP) to form cytidine diphosphate diacylglycerol (CDP-DG). PtdIns is finally formed by exchange of the nucleotide with free inositol by the enzyme CDP-DG inositol phosphatidyltransferase.

DG and PtdA can both supply arachidonate for prostaglandin synthesis (Irvine, 1982 and see Section 1.6) since most of the PtdIns molecules in mammalian tissues and those of its metabolic precursors and products contain arachidonate at position 2.

DG is now accepted as a second messenger responsible for the transmembrane control of protein phosphorylation through the activation of protein kinase C (Nishizuka, 1983). Protein kinase C is found in various tissues in mammals and other eukaryotic organisms (Kuo et al., 1980). The enzyme is widely, although not homogeneously, distributed among tissues. Protein kinase C is a serine- and threonine-specific protein kinase that is dependent upon calcium and phospholipid for activity (Takai et al., 1979). However at physiological calcium concentrations DG is required for activity (Nishizuka, 1984). The enzyme has been purified to homogeneity from a soluble fraction of rat brain (Kikkawa et al., 1982) and more recently the complete primary structure of the 77 kDa protein kinase C has been deduced by recombinant DNA techniques (Parker et al., 1986). This has led to the identification of a new family of protein kinase C-related genes in bovine, human and rat tissues. The alpha-, beta-, and gamma-type protein kinase C sequences are highly homologous, include a kinase domain, and potential calcium-binding sites, and they contain interspersed variable regions (Parker et al., 1986). It is possible that additional members of this gene family may exist and have yet to be isolated.

From studies on protein kinase C in vitro, it has become apparent that those phorbol esters (e.g. TPA) capable of tumour promotion can mimic the effect of DG in enzyme activation (Castagna et al., 1982). More recently, other structurally related and unrelated tumour promoters have also been shown to activate protein kinase C in vitro

(Fujiki et al., 1984; O'Brian et al., 1985). The implication is that these tumour promoters elicit their characteristic responses through protein kinase C. Indeed, there is evidence that phorbol esters do act via high affinity binding sites to provoke cellular responses (Collins and Rozengurt, 1982) and there is both indirect and direct evidence that identifies this high affinity phorbol ester receptor as a protein kinase C itself (Parker et al., 1986).

However, phorbol esters may sometimes be unsuitable for studies of the physiological activation of protein kinase C, since DG is present only transiently in membranes, while phorbol esters are only slowly degraded. Therefore phorbol ester may extend a usually limited phase of cellular response, thereby distorting the normal sequence of events.

Protein kinase C has been postulated to modulate ion conductance by phosphorylating membrane proteins such as ion channels, pumps and ion exchange proteins (Nishizuka, 1986). It may also have a role in the extrusion of Ca^{2+} immediately after its mobilization into the cytosol (by e.g. InsP_3) and the Ca^{2+} transport ATPase is a possible target of this protein kinase (Drummond, 1985; Albert and Tashjian, 1985).

Similarly, a possible role of protein kinase C is activating Na^+ -transport ATPase in peripheral nerve has been suggested. The Na^+/H^+ exchange protein appears to be another target that is activated by phorbol ester or by membrane-permeable DGs and, thus protein kinase C may also function

to increase cytoplasmic pH (Burns and Rozengurt, 1983; Moolenaar et al., 1984a).

A major function of protein kinase C appears to be intimately related to negative feedback control of cell surface receptors, termed down-regulation. The stimulation of the α_1 -adrenergic receptor is well known to induce inositol phospholipid breakdown. Recent evidence from hepatocytes and smooth muscle (Corvera and Garcia-Sainz, 1984; Leeb-Lundberg et al., 1985) strongly suggests that protein kinase C exerts negative feedback control of the receptors of adrenaline and angiotensin II. Similar findings have been suggested for the receptors coupled to inositol phospholipid breakdown in other cells e.g. platelets (Rittenhouse and Sasson, 1985) pituitary cells (Drummond, 1985) and 3T3 cells (Brown et al., 1987).

Down-regulation by protein kinase C also extends, for example, to the EGF receptor, where protein kinase C phosphorylates the receptor with a concomitant decrease in both its tyrosine-specific protein kinase and growth factor-binding activities (Brown et al., 1984; Hunter et al., 1984). Phorbol esters markedly reduce EGF binding in many mitogenically responsive cell types (Lee and Weinstein, 1978; King and Cuatrecasas, 1982). However, activation of protein kinase C by phorbol esters does not inhibit either the autophosphorylation of PDGF receptor molecules at tyrosine residues or the hydrolysis of membrane polyphosphoinositides by PDGF (Sturani et al., 1986).

1.1.4.1 The role of InsP_3 and DG in cell proliferation

The two signal pathways stimulated by the second messengers InsP_3 and DG, namely Ca^{2+} mobilization and protein kinase C activation, may also play roles in long-term responses such as gene expression and cell proliferation. For example, the two pathways are both essential and act synergistically to cause DNA synthesis in macrophage-depleted human peripheral lymphocytes (Kaibuchi et al., 1985; Nishizuka, 1986). However, in order to stimulate rapid cell proliferation, some growth factor is still needed, indicating that an additional signal is involved in eliciting full activation of cell proliferation. For example, phorbol esters and a growth factor (e.g. PDGF) have been known for many years to act in concert to stimulate cell proliferation (Mastro and Mueller, 1974). Insulin is needed in addition to phorbol ester for the growth response of Swiss 3T3 cell lines (Rozengurt et al., 1984). EGF appears to provoke inositol phospholipid breakdown in only a few tissues, such as human A431 cells (Sawyer and Cohen, 1981). However, several growth factors including PDGF (Habenicht et al., 1981) induce rapid breakdown of inositol phospholipids.

Phorbol esters are known to induce gene expression of several enzymes e.g. ornithine decarboxylase (Jetten et al., 1985). A relationship between the phosphoinositide-protein kinase C pathway and the expression of the c-myc protooncogene (see Section 1.2) has been suggested by the observation that PMA stimulates the expression of c-myc in Balb/c-3T3 cells

(Kelly et al., 1983). Further, control of gene expression at the level of transcription has been demonstrated for c-fos protooncogene (Greenberg and Ziff, 1984).

Phorbol esters (e.g. TPA) are also known to induce arachidonic acid (AA) release from cellular phospholipids and the release of cyclooxygenase and lipoxygenase metabolites synthesized in many cell types including fibroblasts (Butler-Gralla et al., 1983 and see Section 1.1.6).

1.1.4.2 The role of GTP and its binding proteins in inositol lipid metabolism

G-proteins respond when a receptor protein is stimulated, by releasing guanosine diphosphate (GDP) and binding guanosine triphosphate (GTP). In its GTP-bound conformation the protein can regulate the function of an effector, e.g. phospholipase C (for the as-yet-uncharacterised G_p -protein). Hydrolysis of bound GTP to GDP terminates the regulatory effect of a G-protein (Bourne, 1986).

There are now known to be at least three related families of GTP binding proteins: the G proteins; the ras proteins; and the elongation factors (such as bacterial EF-Tu) and related proteins involved in protein synthesis (Bourne, 1986).

The heterotrimeric G-proteins contain three distinct chains: an α -chain that binds GTP and determines the specificity of the proteins for its detector and effector, and the β/γ complex that probably serves as an anchor to the cytoplasmic face of the plasma membrane (Northrup, 1985). Molecular cloning studies now show that there are two different

α -chains in G_s , (a G protein that stimulates adenylate cyclase). The two α -chains result from alternate splicing of the transcript of a single gene (Bourne, 1986).

Complementary DNAs encoding α -chains of two other purified G proteins G_i and G_o have also been cloned. G_i mediates inhibition of adenylate cyclase by several hormone and neurotransmitter receptors, (e.g. the muscarinic cholinergic receptor), and is a substrate for pertussis toxin-catalysed ADP-ribosylation, a covalent modification that uncouples G_i from its receptors (Northrup, 1985). The function of G_o is unknown.

It has been suggested, that the receptor-mediated stimulation of inositol lipid metabolism is mediated through a guanine nucleotide regulatory protein called G_p (or N_p) (Gomperts, 1983). Although such a species has not been identified, there is now strong experimental evidence that this process is mediated by a G-protein distinct from the stimulatory or inhibitory G-proteins G_s and G_i respectively (Haslam and Davidson, 1984; Gonzales and Crews, 1985; Houslay et al., 1986).

The ras protein family includes two RAS proteins that control adenylate cyclase in yeast and three p21 proteins that are the products of the mammalian ras protooncogenes, Ha-ras, Ki-ras and N-ras (Harvey, Kirsten and Normal) (Bourne, 1986).

Wakelam et al. (1986) reported that the controlled expression of p21^{N-ras} in NIH 3T3 fibroblasts leads to the increased coupling of certain growth factor receptors (e.g.

for bombesin) to stimulated inositol phosphate production. They proposed that the N-ras protooncogene encodes a protein which couples the receptors for certain growth factors to the stimulation of phospholipase C, and thus may be the putative G_p or a functionally related protein.

1.1.4.3 Inositol lipid metabolism-linked receptors in 3T3 cells

1.1.4.3.1 Vasopressin

The neurohypophysial hormone vasopressin, is a nonapeptide and has been documented by Rozengurt et al. (1979) to increase DNA synthesis in Swiss 3T3 cells. Vasopressin binds to specific, saturable receptors in Swiss 3T3 cells that are of the pressor (V_1) type as judged by: the relative potency of vasopressin-related peptides to elicit various cellular responses, including mitogenesis (Rozengurt et al., 1979); the lack of a direct effect on cyclic AMP levels (V_2 receptors are linked to increases in cAMP) (Rozengurt et al., 1987); and the selective inhibitory effects of vasopressin antagonists which counteract the pressor response of the hormone (Kruszynski et al., 1980; Rodriguez-Pena and Rozengurt, 1986).

Vasopressin stimulates Na^+ influx and Na^+/K^+ ATPase (Mendoza et al., 1980) and increases intracellular pH (Burns and Rozengurt, 1983). The peptide also increases the uptake of deoxyglucose (Dicker and Rozengurt, 1980) and uridine (Becker et al., 1983), induces the activity of the enzyme ornithine decarboxylase (Sreevalsan et al., 1980) and decreases the affinity of cellular receptor for EGF (Rozengurt et al., 1981a). In addition, vasopressin induces phosphoinositide breakdown (Brown et al., 1984) and stimulates the efflux of Ca^{2+}

from an intracellular pool (Lopez-Rivas and Rozengurt, 1984) which leads to a transient increase in intracellular Ca^{2+} concentration (Hesketh et al., 1985). The effect of vasopressin on DNA synthesis is potentiated by the addition of insulin, EGF and/or PDGF (Dicker and Rozengurt, 1981). Vasopressin does not, however, synergize with the phorbol esters (Dicker and Rozengurt, 1981; Collins and Rozengurt, 1982), suggesting a convergence in their mechanisms of action. This possibility is strengthened by the findings of Rodriguez-Pena and Rozengurt (1986) who have shown that vasopressin rapidly stimulates protein kinase C in Swiss 3T3 cells.

1.1.4.3.2 Bombesin

Bombesin is a tetradecapeptide obtained from the skin of certain European species of frog; it is the amphibian equivalent of gastrin-releasing peptide and is structurally related to the neuromedins (McDonald et al., 1979). These peptides are potent mitogens for Swiss 3T3 cells (Minamino et al., 1983; 1984). They bind to high-affinity cell-surface receptors in Swiss 3T3 cells (Zachary and Rozengurt, 1985). A major component of the receptor for peptides of the bombesin family, in 3T3 cells, has been identified as a surface protein $M_r = 75-85$ kDa (Zachary and Rozengurt, 1987). Bombesin elicits a complex array of early biological responses including enhanced phosphoinositide metabolism (Brown et al., 1984; Wakelam et al., 1986), stimulation of Na^+/H^+ antiport activity and mobilization of Ca^{2+} from intracellular stores (Mendoza et al., 1986).

Like vasopressin, it has also been shown to activate protein kinase C, inhibit EGF binding and act synergistically with insulin and other growth factors in increasing DNA synthesis. Rozengurt and Sinnett-Smith (1987) very recently have shown that bombesin and vasopressin can induce the expression of the cellular protooncogenes c-fos and c-myc (see Section 1.2).

Bombesin, however, differs from vasopressin, in its ability to stimulate reinitiation of DNA synthesis in serum-free medium in the absence of any other added growth factor. This contrasts with many other ligands including insulin (Rozengurt et al., 1979; 1981a), vasopressin (Rozengurt et al., 1979; 1981a), phorbol esters (Collins and Rozengurt, 1982), cAMP-increasing agents (Rozengurt, 1982), EGF (Dicker and Rozengurt, 1980), and PGF_{2α} (Jimenez de Asua et al., 1979; Otto et al., 1982), which stimulate DNA synthesis in Swiss 3T3 cells only when added in the presence of other synergistic growth factors. Interestingly, vasopressin and phorbol esters, which modulate diverse cellular functions by a common mechanism (Rozengurt et al., 1981a), or the cAMP-increasing agents (cholera toxin and 8-bromo cAMP) (Rozengurt, 1982a), fail to enhance the maximal level of DNA synthesis induced by bombesin in 3T3 cells. This suggests (Rozengurt and Sinnett-Smith, 1983), that the pathways converge with those utilized by two different classes of mitogenic agents.

However, Rozengurt et al. (1987) have shown that although vasopressin does not increase the basal level of cAMP (like bombesin), or stimulate adenylate cyclase in membrane prepared

from Swiss 3T3 cells, it does enhance cAMP accumulation in response to cholera toxin and forskolin. The modulation of cAMP production by vasopressin was mediated by V_1 receptors (which are not coupled directly to adenylate cyclase). Rozengurt et al. (1987) suggest that this is due to activation of protein kinase C which they propose amplifies cAMP production in Swiss 3T3 cells.

1.1.4.3.3 Prostaglandin $F_{2\alpha}$

Among the biochemical events observed upon stimulation of confluent, resting 3T3 cells, by different mitogens, are rapid changes in surface membrane properties (the pleiotypic response) and, importantly, the metabolism of inositol phospholipid (Michell, 1982). Addition of foetal calf serum, FDGF, or PDGF to Swiss 3T3 cells increases inositol lipid metabolism (Ristow et al., 1980; Habenicht et al., 1981; Berridge et al., 1984a). Sawyer and Cohen (1981) have shown that the addition of EGF to A-431 cells (human epidermoid carcinoma cells) causes a marked increase in inositol lipid metabolism and Ca^{2+} uptake. As has been discussed already, 1,2-diacylglycerol is formed in the breakdown of $PtdIns(4,5)P_2$ and this can lead to the production of arachidonic acid and to prostaglandin biosynthesis. This led Jimenez de Asua and colleagues (Jimenez de Asua et al., 1979; Otto et al., 1982) to look at the effects of a series of structurally closely related prostaglandins on cell division in 3T3 fibroblasts. They found that $PGF_{2\alpha}$, when added to quiescent Swiss 3T3 cells, initiated DNA synthesis and cell proliferation in a small proportion of cells.

Insulin markedly potentiated this effect when added in combination with $\text{PGF}_{2\alpha}$ and together they caused a simultaneous decrease in intracellular cAMP, and an increase in cGMP concentrations, similar to those observed after serum addition. PGE_1 and PGE_2 were found to be less effective than $\text{PGF}_{2\alpha}$ in initiating DNA synthesis (Taylor and Polgar, 1977; O'Farrell et al., 1979). Further, it was found that the mitogenic effect in mouse fibroblasts is selective for $\text{PGF}_{2\alpha}$ among naturally occurring prostaglandins (Jimenez de Asua et al., 1983) but PGE_1 and PGE_2 at concentrations that are not mitogenic have a synergistic effect in DNA synthesis stimulated by $\text{PGF}_{2\alpha}$ (Otto et al., 1982).

Macphee et al. (1984) showed that in confluent, resting Swiss 3T3 cells, $\text{PGF}_{2\alpha}$ stimulated the incorporation of $[^{32}\text{P}] - \text{PO}_4$ into PtdIns and increased the cellular content of 1,2-DG. These effects were not seen with EGF or PGE_1 .

1.1.4.3.4 PDGF-linkage with inositol phospholipid hydrolysis

PDGF, which as discussed above, can activate tyrosine kinase activity (see Section 1.1.3.1.2) has also been shown to cause an increase in the formation of DG (Habenicht et al., 1981) and InsP (Berridge et al., 1984a). One area of this project is aimed at studying the mechanism of action of PDGF, as it has yet to be documented whether inositol lipid metabolism is an indirect response to PDGF receptor activation, mediated, perhaps, via tyrosine kinase, or if receptor activation is directly linked to inositol phospholipid hydrolysis.

1.5 Cyclic-AMP

Most cells possess at least two major classes of receptors for transducing information across the plasma membrane. One class, as has been discussed in Section 1.1.4, accelerates the breakdown of inositol phospholipids, leading to the generation of $\text{Ins}(1,4,5)\text{P}_3$, which mobilizes intracellular Ca^{2+} , and DG, which activates protein kinase C. The other class of receptors influences cell activity through the altered synthesis of cAMP.

The enzyme responsible for the formation of cAMP is adenylate cyclase. It is present in the plasma membrane of most cells. In the presence of Mg^{2+} ions this enzyme catalyzes an intramolecular condensation of ATP in the cytoplasm to produce cAMP.

Cyclic AMP has the property of activating several protein kinases. It is now accepted as a second messenger in mediating the effects of a number of hormones and neurotransmitters (e.g. adrenaline and thyrotrophin). These, following the interaction with specific receptors, regulate adenylate cyclase by allowing GTP-regulatory proteins to interact and control the enzyme's activity (Rodbell, 1980). The GTP-regulatory protein (see Section 1.1.4.2) can either be stimulatory (Ns) or inhibitory (Ni) in nature, thereby increasing or decreasing, respectively, the production of cAMP.

Cholera toxin acts to increase the production of cAMP in a cell by ADP-ribosylating the α -subunit of N_s . This results in the inhibition of the GTPase activity of N_s and converts GTP to a superactive nucleotide like its non-hydrolysable analogues (e.g. GMP-P(NH)P) (Milligan and Klee, 1985; Northrup, 1985).

In contrast, pertussis toxin also stimulates the production of cAMP, but in this case, by ADP-ribosylating the α -subunit of N_i . This is thought, ultimately, to lead to blockade of the N_i -GDP complex, very likely by inhibiting its dissociation to give free N_i and GDP (Northrup, 1985).

1.5.1 Cyclic AMP-linked receptors

The possibility that cAMP may regulate the proliferative response of quiescent fibroblasts has been the subject of a large and controversial literature (e.g. Hammarstrom, 1982; Rozengurt et al., 1981b).

Cyclic AMP is known to act synergistically with insulin and other growth promoting agents in stimulating confluent and quiescent cultures of Swiss 3T3 cells to reinitiate DNA synthesis and cell division (Rozengurt, 1982b). The levels of cAMP in these cells can be increased by cholera toxin (Rozengurt et al., 1981b), adenosine agonists (Rozengurt, 1982b), cAMP analogues or E-type prostaglandins (Minna and Gilman, 1973; Rozengurt, 1982a).

Recently several investigators have found that there is "cross-talk" between the cellular signalling pathways, cAMP and inositol phospholipid hydrolysis. Both systems are

controlled through guanine nucleotide regulatory proteins (G-proteins) to lead ultimately to specific activation of either cAMP-dependent protein kinase or Ca^{2+} /phospholipid-dependent protein kinase C. Rozengurt et al. (1987) demonstrated that protein kinase C activation of phorbol esters, in Swiss 3T3 cells, was coupled to cAMP production via a pertussis toxin sensitive substrate (which they assume to be a G protein). Furthermore, Yoshimasa et al. (1987) showed that in frog erythrocytes TPA produces phosphorylation of the catalytic subunit of adenylate cyclase. Moreover purified protein kinase C can directly phosphorylate in vitro, the catalytic unit of adenylate cyclase purified from bovine brain (Yoshimasa et al., 1987).

Activation of protein kinase C can also inhibit the accumulation of cAMP promoted by activators of adenylate cyclase in different cell types (Rozengurt et al., 1987). Inhibition is frequently caused by phosphorylation and inactivation of receptors linked to adenylate cyclase.

1.5.1.1 The prostaglandins

E-type prostaglandins are potent stimulators of adenylate cyclase in a number of tissues (Samuelsson et al., 1978). An increase in cellular levels of cAMP, in response to E type prostaglandins has been measured in a variety of cultured cells (Samuelsson, et al., 1978) including Swiss 3T3 cells (Rozengurt et al., 1983b). Some reports have indicated that PGE_1 , at high concentrations, increased cAMP levels and inhibited the initiation of DNA synthesis in Swiss 3T3 cells (e.g. Hammarström, 1982). However other workers have

demonstrated that in the presence of insulin PGE_1 stimulates DNA synthesis (Rozengurt et al., 1983b). These workers also found that the inhibitory effects produced by adding high concentrations of PGE_1 on the initiation of DNA synthesis in Swiss 3T3 cells were not mediated by cAMP and suggested that they were non-specific effects.

PDGF causes a marked accumulation of cAMP in the presence of phosphodiesterase inhibitors (Rozengurt et al., 1983c). However, this increase in cAMP is much slower than the elevation of cAMP observed in the presence of e.g. PGE_1 , which results from the receptor-mediated stimulation of adenylate cyclase (Rozengurt et al., 1983c).

It is thought that PDGF stimulates the breakdown of AA by cyclooxygenase (see next section) to yield metabolites such as prostaglandins which in turn stimulate adenylate cyclase. Indeed Habenicht et al. (1985), have suggested that PDGF greatly activates cyclooxygenase, an effect which appears to be mediated by both activation and rapid de novo synthesis of the enzyme. The addition of AA has also been shown to have a stimulatory effect on cAMP in 3T3 cells (Claesson et al., 1977). This effect of AA might be mediated, at least in part, through the formation of prostacyclin (PGI_2) (Claesson et al., 1977).

1.5.1.2 Adenosine agonists

Adenosine binds to cell surface receptors of a variety of cell types and either stimulates or inhibits the activity of adenylate cyclase (Fain and Malbon, 1979). The most

potent of the adenosine analogues, that binds selectively to stimulatory receptors is 5'-N-ethylcarboxamide-adenosine (NECA) (Londos et al., 1980). Addition of NECA caused a rapid increase in the levels of cAMP, an effect markedly potentiated by Ro20-1724 (cAMP phosphodiesterase inhibitor) (Rozengurt, 1982b). Subsequently, the adenosine analogue was shown to stimulate [^3H] -thymidine incorporation into the acid-insoluble material in cultures of Swiss 3T3 cells maintained in the presence of Ro20-1724 and insulin. The increase in cAMP levels and stimulation of DNA synthesis produced by addition of NECA to Swiss 3T3 cells were completely and selectively blocked by aminophylline (Rozengurt, 1982b). (adenosine antagonist). Thus the mitogenic activity of NECA closely parallels its ability to increase the intracellular levels of cAMP.

1.6 Arachidonic acid liberation and metabolism

Arachidonic acid liberation

As mentioned previously both DG and PtdA can supply arachidonate for prostaglandin and leukotriene synthesis. The mechanisms underlying arachidonic acid liberation have been extensively investigated. For example, in blood platelets, arachidonic acid (AA) is predominantly found esterified in the sn-2 position of membrane phospholipids, particularly phosphatidylcholine (PtdCho), phosphatidylethanolamine (PtdE) and phosphatidylinositol (PtdIns). Four major routes of AA liberation have been postulated (Figure 3).

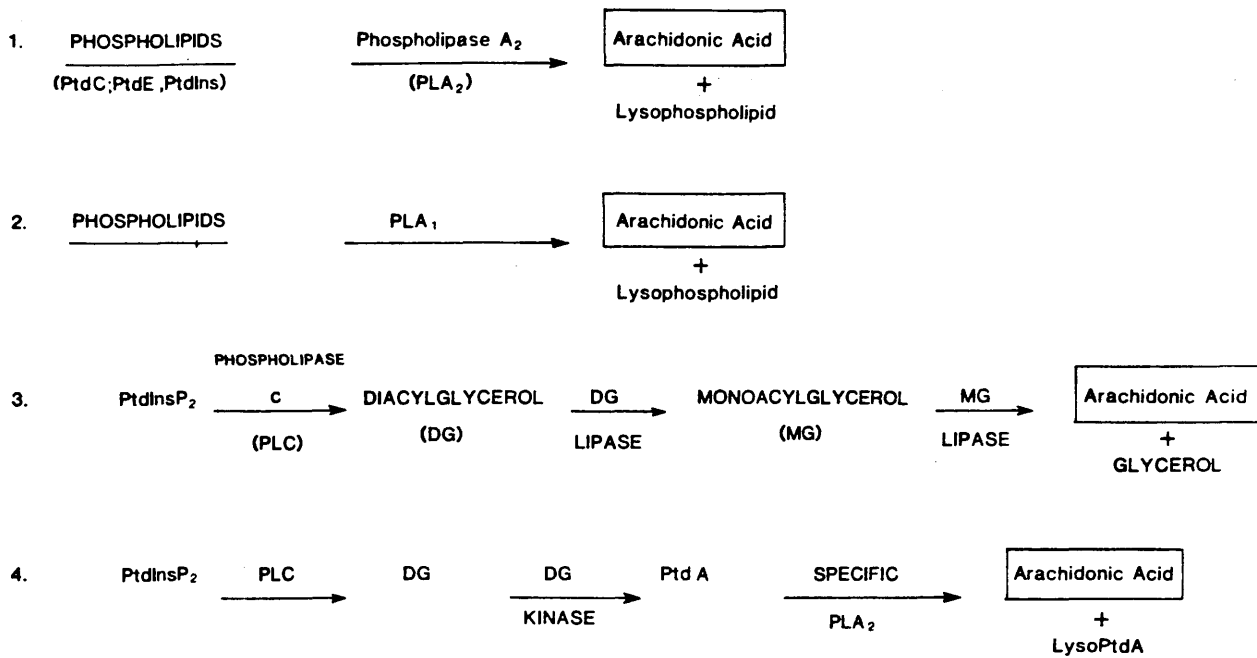


Figure 3: The possible pathways of arachidonate liberation from membrane phospholipids

Studies by Shier (1979, 1980) have shown that cultured cell lines, such as 3T3 mouse fibroblasts, contain high levels of activatable cellular enzymes capable of releasing labelled AA which had been biosynthetically incorporated into the phospholipids of the cells. The major enzyme activated by PDGF in these cells is phospholipase A₂ (PLA₂) (Shier and Trotter, 1980). Phospholipases of the A₂ type are considered to be Ca²⁺-calmodulin-dependent and, as such, susceptible to activation by an increase in intracellular free Ca²⁺ (Pollock et al., 1986).

Anti-inflammatory glucocorticoids, such as hydrocortisone and dexamethasone, are thought to inhibit PLA₂, indirectly, by stimulating the production of phospholipase inhibitory proteins, e.g. lipomodulin (Hirata et al., 1980; Hirata et al., 1982); macrocortin (Blackwell et al., 1978); renocortin (Rothhut et al., 1983). Steroids can also produce a more rapid effect which may be due to a "direct" phospholipase activity. This effect was illustrated by Kato et al. (1985), who found that the hydrolysis of [³H] PtdCho by PLA₂ could be inhibited by incubating the PLA₂ with dexamethasone for 3-10 minutes.

Recently, many studies have focussed on identifying the phospholipase inhibitory proteins. Several proteins, collectively known as "lipocortins" have been described which inhibit PLA₂ (e.g. Touqui et al., 1986).

Interestingly, the cDNA sequence of rat lipocortin I and murine p36 (the 36 kDa subunit of calpactin I - see Section 1.1.3.1) have been determined and show an overall

homology of 50% at the amino acid level (Huang et al., 1986; Saris et al., 1986). p36, As already discussed is a widely distributed cellular protein (0.1 - 0.4% in fibroblasts, Hutton, 1986). In the guise of the porcine intestinal protein calpactin I, p36 has been postulated to be a substrate for the tyrosine kinase associated with pp60^{v-src} (see Section 1.2), EGF and PDGF receptors. The p36 subunit is phosphorylated, in vivo, on both tyrosine and serine residues, the latter by protein kinase C (Hutton, 1986).

The homology of p36 with lipocortin I and its ability to inhibit pancreatic PLA₂ suggests a possible, but unproven, role as an inhibitor of intracellular phospholipases (Huang et al., 1986). Also, the observation that the 40 kDa-protein kinase C substrate, whose phosphorylation state changes dramatically upon platelet activation, may partially cross-react with an antibody raised to rat renal lipocortin, is of great interest (Touqui et al., 1986). Touqui et al. (1986) suggest that protein kinase C phosphorylates the anti-PLA₂ protein (40 kDa) and suppresses its anti-PLA₂ activity. This, in agonist-stimulated platelets, combined with a rise in $[Ca^{2+}]_i$, via InsP₃ formation leads to optimal activity of PLA₂.

PLA₂ activity can be stimulated by an array of toxins (Shier, 1980). One such toxin is melittin, a 26-residue peptide, which is the most abundant component of wasp venom (Argiolas and Pisano, 1983). Melittin has been shown to

increase the activity of the $\text{Na}^+\text{-K}^+$ pump in quiescent 3T3 cells (Rozengurt et al., 1981c). It also stimulates DNA synthesis in quiescent mouse cells, acting synergistically with insulin, EGF and PDGF. In contrast it does not act synergistically with either phorbol esters or vasopressin (Rozengurt et al., 1981c).

1.1.6.1 Arachidonic acid metabolism

Cyclooxygenase products

Arachidonic acid and certain other polyunsaturated fatty acids (Needleman et al., 1986) may be transformed into prostaglandins by the enzyme prostaglandin endoperoxide synthase (PES); for review see Needleman et al. (1986). Figure 4 shows formation, by the PES family of enzymes, of the various prostaglandins and thromboxanes.

The enzyme cyclooxygenase can be inhibited using the non-steroidal anti-inflammatory agents, such as aspirin (which irreversibly binds to the enzyme) (Pace - Asaak and Smith, 1983) and ibuprofen (which reversibly binds to the enzyme. Indomethacin is a relatively non-specific blocker of cyclooxygenase whereas flurbiprofen (one of the ibuprofen class of drugs) has been shown to be more specific for cyclooxygenase (Brodgen et al., 1979). Certain acetylenic fatty acids such as eicosa-5,8,11,14-tetraynoic acid (ETYA) also inactivate cyclooxygenase possibly by acting as suicide substrates (Needleman et al., 1986).

Numerous studies have shown that, in vivo as well as in vitro, prostaglandins can regulate the rate of proliferation and differentiation of certain mammalian cells (e.g. Powles

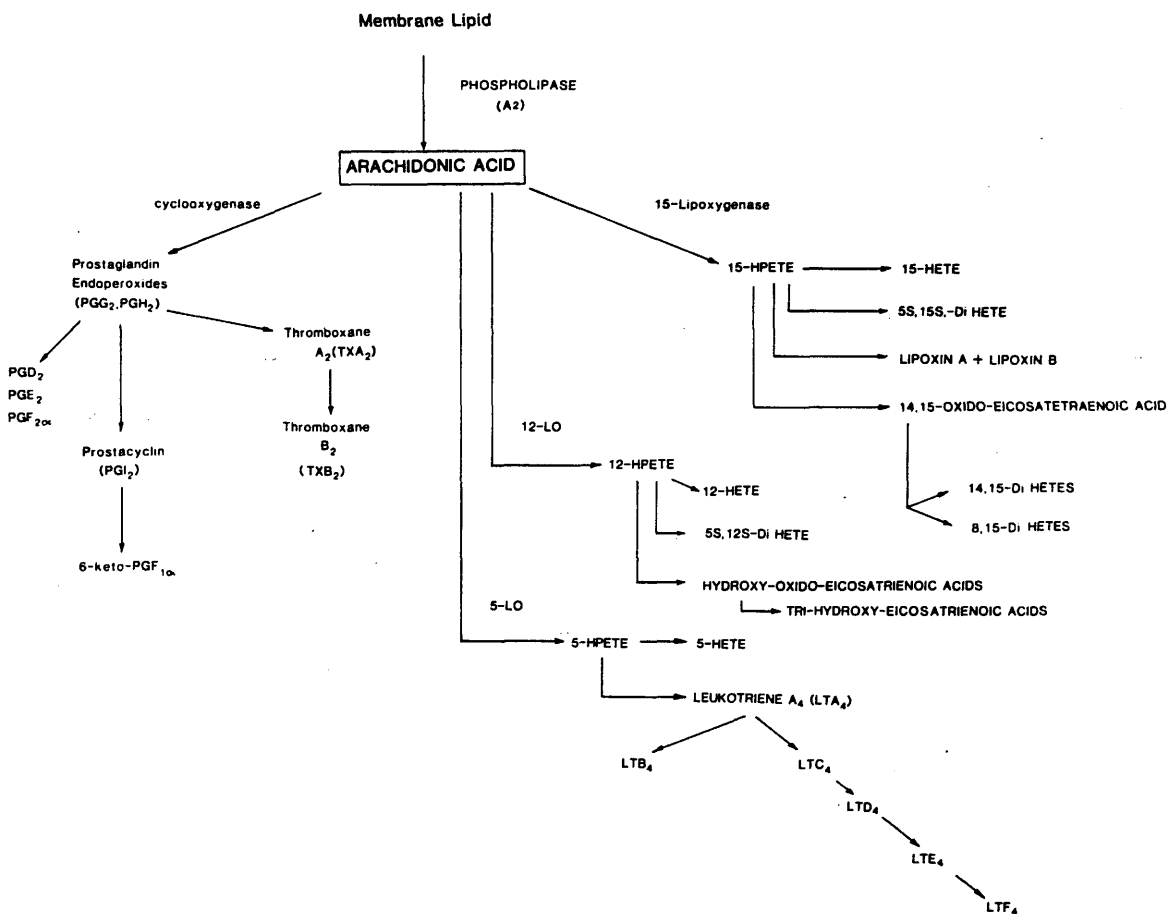


Figure 4: The metabolic pathways involved in the degradation of arachidonic acid

et al., 1982). In a number of malignant tumours the synthesis and release of prostaglandins is markedly increased (Levine, 1982). The production and release of PGE_1 , PGE_2 or $\text{PGF}_{2\alpha}$ is increased in several cell lines transformed by chemicals or oncogenic viruses, and proliferation in these cell lines is reduced when prostaglandin synthesis is inhibited (Hammarstrom, 1977). Furthermore TPA and several other growth factors and tumour promoters, increase the synthesis of PGE_1 , PGE_2 and/or $\text{PGF}_{2\alpha}$ (Levine, 1982).

PGE_1 and PGE_2 have been shown to enhance the mitogenic effect of $\text{PGF}_{2\alpha}$ alone, or in combination with insulin in 3T3 cells (Otto et al., 1982). This group of workers also showed that the addition of EGF with $\text{PGF}_{2\alpha}$ enhanced the stimulatory properties of EGF with respect to DNA synthesis. This was further enhanced by the addition of insulin. In contrast PGE_1 or PGE_2 had no such effect. Macphee et al. (1984) showed, as mentioned later, that $\text{PGF}_{2\alpha}$ rapidly stimulated the turnover of PtdIns and increases the level of DG within minutes of its addition to resting Swiss 3T3 cells. In contrast, EGF which is mitogenic for these cells (Macphee et al., 1984) did not increase PtdIns turnover. PGE_1 did not potentiate this effect of $\text{PGF}_{2\alpha}$, even though it interacts with $\text{PGF}_{2\alpha}$ to synergistically increase DNA synthesis. This indicates that the synergistic interaction of $\text{PGF}_{2\alpha}$ and PGE_1 occurs at a biochemical event different from PtdIns turnover.

1.1.6.2 Lipoxygenase products

Arachidonic acid can also be metabolised by a different group of enzymes known as the lipoxygenases (Figure 4).

The formation of 12-S-hydroperoxy-5,8,10,14-eicosatetraenoic (12-HPETE) in human platelets was the first lipoxygenase-type reaction reported in animals (Hamberg and Samuelsson, 1974).

More recently, leukotrienes (LT) were isolated and found to be the products of a lipoxygenase (1.0.) acting specifically at the C-5 position of arachidonic acid (Samuelsson, 1983). 5-Lipoxygenase catalyzes the dioxygenation of AA at C-5 and initiates the synthesis of leukotrienes. AA is first transformed to (5S)-5-hydroperoxy-(E,Z,Z,Z)-6,8,11,14-eicosatetraenoic acid (5-HPETE). This is rapidly metabolized, by what is thought to be a peroxidase enzyme, to (5S)-5-hydroxy-(E,Z,Z,Z)-6,8,11,14-eicosatetraenoic acid (5-HETE), or by an enzymatic hydrolysis to (5S, 6S)-5(6)-oxido-(E,E,Z,Z)-7,9,11,14-eicosatetraenoic acid (leukotriene A_4 , LTA_4) (Borgeat et al., 1985).

LTA_4 is highly unstable and is rapidly converted to LTB_4 by a hydrolase or to LTC_4 by glutathione-S-transferase. LTD_4 is formed from LTC_4 via the γ -glutamyl-transpeptidase enzyme. Further metabolism of LTD_4 , by a peptidase, leads to the formation of LTE_4 . Reincorporation of a glutamyl moiety by γ -glutamyl-transpeptidase on LTE_4 yields LTF_4 (Figure 4, Borgeat et al., 1985).

Little work on the effects of lipoxygenase metabolites in fibroblasts has been carried out. Baud et al. (1987) studied the role of leukotrienes on fibroblast growth and

its modulation by the endogenous synthesis of PGE_2 . LTB_4 has been shown to have no significant direct effect on fibroblast proliferation. However, the sulfidopeptide leukotriene series (LTC_4 , D_4 and E_4) was demonstrated to stimulate fibroblast proliferation (Baud et al., 1987) in the presence of a cyclooxygenase inhibitor (indomethacin, aspirin and ibuprofen).

Another group of compounds carrying a conjugated triene unit, but derived from 15-lipoxygenase, has been reported (Borgeat et al., 1985) (15S)-15-hydroperoxy-(Z,Z,Z,E)-5,8,11,13-eicosatetraenoic acid (15-HPETE) is the product of the action of 15- LO on AA and is rapidly reduced into 15-HETE (Borgeat and Samuelsson, 1979; Turk et al., 1982) or metabolized into an unstable allylic epoxide in analogy with the mechanism of formation of LTA_4 from 5-HPETE (Figure 4). The allylic 14,15-epoxy-acid undergoes facile hydrolysis into several dihydroxy-acids (14,15-Di-HETEs and 8,15-Di-HETEs).

Human neutrophils incubated with 15-HPETE and A23187, (a calcium ionophore), form at least two trihydroxylated compounds with a conjugated tetraene structure: 15,16,17(L)-trihydroxy-7,9,11,13-eicosatetraenoic acid (lipoxin A, LXA) and 5(D), 14,15(L)-trihydroxy-6,8,10,12-eicosatetraenoic acid (Lipoxin B, LXB) (Serhan et al., 1984a; 1984b). Analogous products are formed from the 15-hydroperoxy-derivative of eicosapentaenoic acid (Wong et al., 1985). It appears likely that the 5-lipoxygenase participates in the formation of

these compounds (Serhan et al., 1984b). LXA stimulates superoxide anion generation without provoking aggregation when added to human neutrophils (Serhan et al., 1984b). Human natural killer cells exposed to either LXA or LXB are unable to provoke target cell lysis (Ramstedt et al., 1985). In addition, LXA also stimulates contraction of parenchymal strips and microvascular changes (Serhan et al., 1986). Moreover, lipoxins have recently been shown to activate protein kinase C in vitro (Hansson et al., 1986; Serhan et al., 1986). LXA activates protein kinase C and is 30 times more potent than DG and 300 times more potent than AA in this respect. LXB is ten times less potent than LXA in this assay (Serhan et al., 1986).

1.2 Oncogenes and their relationship with growth factors

In 1911, Peyton Rous discovered the first oncogenic virus, the avian sarcoma virus, which is today known as Rous sarcoma virus (RSV). However, it was not until the early 1970's that the gene responsible for rapid oncogenesis (leading to cancer in chickens) was identified. The gene was named 'src' and the protein for which it encodes was isolated by Brugge and Erikson (1977). It was designated p60src because its molecular mass is 60 kDa.

Since then, a number of viruses have been discovered which produce some type of cancer in experimental animals. Several studies indicated that the viral genome was in whole or in part incorporated into the genome of the host cell and eventually produced transformation (for review see Bishop,

1985). In the last few years, the molecular biology of carcinogenesis has seen extraordinary advances largely due to the knowledge acquired on the mechanisms whereby certain oncogenic viruses, the acute transforming retroviruses, have an oncogenic gene (e.g. src): only if this gene is present does retroviral infection lead to transformation of cells (Bishop, 1983).

Retroviruses have an RNA genome and they use reverse transcriptase to make a complementary DNA copy of this RNA, which then becomes double stranded DNA, and is eventually incorporated into the genome of the host cell. During this process the retrovirus picks up a cellular gene and incorporates it into its own 'provirus' DNA. Somewhere along this path the host gene (protooncogene) mutates and gives rise to an oncogenic gene (oncogene) (Ochoa, 1985). The virus now becomes oncogenic. It is important to understand that viral oncogenes are mutants of normal cellular genes (protooncogenes). The normal function of the protooncogene is unknown, although it has been assumed that protooncogenes participate in the regulation of cell proliferation. This view has gained support from recent data that seem to link certain oncogene products (transforming proteins as well as their normal cellular homologs) to different compartments along the mitogenic pathway of a normal cell.

The biochemical mechanisms involved in the action of oncogenes are proposed to fall into three categories listed in Table 2.

Table 2: The biochemical mechanisms involved in the action of oncogenes (Bishop, 1985)

1. Phosphorylation (with both proteins and phospholipids as potential substrates - Hunter and Cooper, 1985).

The transforming protein may be a factor that elicits phosphorylation, such as a ligand that binds to a receptor on the cell surface; it may be the catalytic kinase itself; or it may act on phosphorylation, at a distance, by regulating adenylate cyclase and thus the activity of protein kinases controlled by cAMP. In each of these instances, the primary action occurs at the plasma membrane: the responsible protein strikes at the cell surface, spans the plasma membrane, or resides at the inner surface of the membrane.

2. Initiation of DNA synthesis (Martin, 1981)

Unrestrained synthesis of DNA is an inevitable component of the neoplastic phenotype. Some transforming proteins allegedly elicit this property by acting directly to initiate DNA synthesis.

3. Regulation of transcription (Kingston et al., 1985)

Several of the transforming proteins may influence transcription from cellular genes, either by stimulation or by inhibition. They could do so by interacting with other proteins, with promoters for transcription or with enhancers.

Twenty or more oncogenes are now known. Their oncogenicity is due to the products of the gene expression, namely, proteins encoded by the oncogenes. As has been discussed, these proteins have a biochemical function. Many of the products of retroviral oncogenes have a tyrosine kinase activity (Hunter and Cooper, 1985). This activity leads to self-phosphorylation as well as to phosphorylation of other proteins (Table 2). This property is, therefore, one which is shared with certain of the growth factor receptors (see Section 1.1.3.).

All of the protein-tyrosine kinases that have been studied, to date, are related to catalytic domains of about 30 kDa in which reside a series of amino acid sequences that are a common feature of protein kinases in general, and of protein-tyrosine kinase in particular (Hunter and Cooper, 1985). The members of this enzymatic family have so far taken either of two forms: transmembrane receptors for growth factors or cytoplasmic proteins affiliated with membranes (Hunter and Cooper, 1985, Yarden et al., 1986). Retroviral transforming proteins are found in both classes.

Further evidence linking oncogenes with growth factors has emerged from studies on the PDGF structure. A partial amino sequence analysis of separated PDGF polypeptide chains, denoted A and B (Johnsson et al., 1982), has shown that the N-terminal 109 amino acids are virtually identical with the predicted sequence of the transforming protein p28^{v-sis} of simian sarcoma virus (residues 67-175) (Doolittle et al., 1983; Waterfield et al., 1983).

Furthermore, there is evidence for the internal structural homology within the PDGF molecule. Of the 75 amino acid residues sequenced in the A-chain about 60% are homologous to the B-chain (Johnsson et al., 1984). Following its synthesis the 28 kDa product of v-sis assembles into a homodimer (Robbins et al., 1983). Some cells appear to release a homodimer of v-sis, whose structure and activity resemble those of PDGF (Johnsson et al., 1985). Also, ssv-transformed cells contain, in their cytoplasm, a growth factor whose activity is neutralized by PDGF antibodies (Deuel et al., 1983). The use of PDGF antibodies has also indicated that FDGF has similar characteristics of PDGF, as FDGF, isolated from the conditioned medium of SV-40 transformed BHK cells, binds to a PDGF antibody (Stroobant et al., 1985).

Not only have oncogenes been related to the molecular structure of a growth factor also, it has been found that oncogenes can be related to the structure of the receptor for a growth factor. For example, the product of v-erb B (the transforming protein of avian erythroblastosis virus - AEV) has been demonstrated by Downward et al. (1984) to be a truncated version of the receptor for EGF.

Very recently, it has been suggested (Wakelam et al., 1986) that oncogenes may also encode proteins which are very similar normal cellular proteins involved in other types of receptor activated signal transduction mechanisms. Wakelam et al. (1986) demonstrated that the expression of

normal p21^{N-ras} in NIH 3T3 fibroblasts leads to the coupling of certain growth factor receptors (e.g. bombesin) to stimulated inositol phosphate production. They proposed that the N-ras protooncogene encodes a protein which couples the receptors for certain growth factors to the stimulation of phospholipase C. Thus, they concluded, N-ras p21 may be the putative G_p (G protein which links receptor activation to PLC).

A particularly intriguing possibility is that the activated 'ras' gene (from Kirsten and Harvey sarcoma viruses) may transform cells by causing an uncontrolled stimulation of PLC. The normal ras gene can both bind and hydrolyse GTP, but on activation by a point mutation at codon 12, the resulting oncogenic protein can still bind GTP, although its ability to hydrolyse the nucleotide is severely impaired (Wakelam et al., 1986). If ras were involved in the hydrolysis of PtdIns(4,5)P₂, the loss of GTPase activity would imply that the oncogenic protein might continue to activate the formation of Ins(1,4,5)P₃ and DG in an uncontrolled way, independently of the presence of growth factors (Wakelam et al., 1986).

The mechanism by which growth factor-induced mitogenic signals are transmitted further into the cell to the nucleus are still largely unknown. Recent data have introduced the idea of cellular protooncogenes (e.g. c-myc) which carry the mitogenic signals induced by growth factors to the nucleus where DNA synthesis is initiated.

The gene, c-myc, is transcriptionally silent in G₀ until activated by a mitogen. Recently, pentadecadeoxyribonucleotide complementary to the initiation codon and four downstream

codons of human c-myc mRNA has been reported to specifically inhibit the proliferation of the human leukaemic cell line HL-60 (Wickstom et al., 1986). Heikkila et al. (1987) reported that this same c-myc complementary oligonucleotide (antisense) inhibits mitogen-induced c-myc protein expression in human T-lymphocytes and prevents S phase entry. Interestingly the c-myc antisense treatment did not inhibit G_0 to G_1 traverse.

Addition of PDGF to quiescent BALB/c 3T3 cells for 2 hours induces a several-fold increase in the expression of c-myc mRNA, as does addition of mitogens to mouse lymphocytes (Kelly et al., 1983). Since the c-myc product is a nuclear protein (Abrams et al., 1982) one might envisage a functional role for it in the regulation of the expression of the genetic program that controls cell proliferation. The c-myc gene, as transduced by several retroviruses, transforms a whole array of target cells of different histogenic origins (Graf and Beug, 1978). Abnormal or amplified expression of the c-myc gene has also been implicated in human malignancies e.g. Burkitt's lymphoma (Dalla Favera et al., 1982). It may be that the post receptor pathways of several mitogens converge at the regulation of the myc gene expression and hence this may be the explanation for the wide variety of neoplasias associated with the abnormal expression of this particular oncogene (Armelin et al., 1984).

Another cellular protooncogene c-fos has been found to be rapidly induced by growth factor or mitogens in fibroblasts and hematopoietic cells (Kelly et al., 1983) suggesting that c-fos also may play an important role in the control of cell proliferation.

1.3 The aims of the project

It must be stressed, from the beginning, that it was not the aim of this project to look at mitogenesis in 3T3 cells. Initially it was concerned with following up the experiments of Macphee et al. (1984). Thus, the effects of $\text{PGF}_{2\alpha}$ on inositol lipid metabolism were studied in more detail.

However, in a broader sense, the project was concerned with investigating the effects of arachidonic acid and its metabolites on inositol lipid metabolism, and in particular whether it, and/or its metabolites, plays a role in mediating the action of PDGF on inositol lipid metabolism.

As discussed in previous sections, PDGF can act through the tyrosine kinase activity of its receptor. The PDGF receptor is structurally unrelated to G-protein-linked receptors such as the β -adrenergic receptor (Yarden et al., 1986), which might suggest that its signal transduction pathway does not involve a G protein. However, PDGF also stimulates inositol phospholipid hydrolysis and thus by analogy with other receptors may act through a G protein, (G_p type, possibly), to stimulate phospholipase C. One possibility is that there may be more than one type of PDGF receptor, one which employs a G protein, the other which does not.

There are a number of possible ways in which arachidonate metabolism might be envisaged to play a role in the mitogenic-

and inositol lipid cycle-stimulating action of PDGF. One theoretical model is illustrated in Figure 5. This proposes that PDGF binding to its receptor stimulates its tyrosine kinase activity, which leads to the phosphorylation and, hence, the inhibition of a lipomodulin (p36 type protein). The inhibition of this inhibitory lipomodulin would allow PLA_2 to hydrolyse phospholipids in the plasma membrane to liberate arachidonic acid (AA). The AA could then, itself, be metabolised by cyclooxygenase to yield PGE_2 and $\text{PGF}_{2\alpha}$, on the one hand, and PGI_2 on the other. It is thought that PGI_2 causes a rise in cAMP (Claesson et al., 1977) and this could contribute to mitogenesis. The $\text{PGF}_{2\alpha}$ and PGE_2 , in contrast, could induce an increase in 1,2-diacylglycerol (DG) and thus may also contribute to mitogenesis.

PDGF is a more powerful mitogen than, for example, $\text{PGF}_{2\alpha}$. This is presumably due to the ability of PDGF to act through more than one transduction pathway e.g. tyrosine kinase and inositol lipid metabolism. The resultant stimulatory effects of these pathways could be synergistic in stimulating mitogenesis.

Another possibility (Figure 5) is that PDGF receptor activation is coupled directly to phospholipase C activation. This enzyme hydrolyses $\text{PtdIns}(4,5)\text{P}_2$ to yield $\text{Ins}(1,4,5)\text{P}_3$ and DG. An increase in DG formation activates protein kinase C which leads to cell proliferation and it also increases cellular pH (a signal associated with mitogenesis). $\text{Ins}(1,4,5)\text{P}_3$ formation leads to intracellular Ca^{2+} mobilization. This

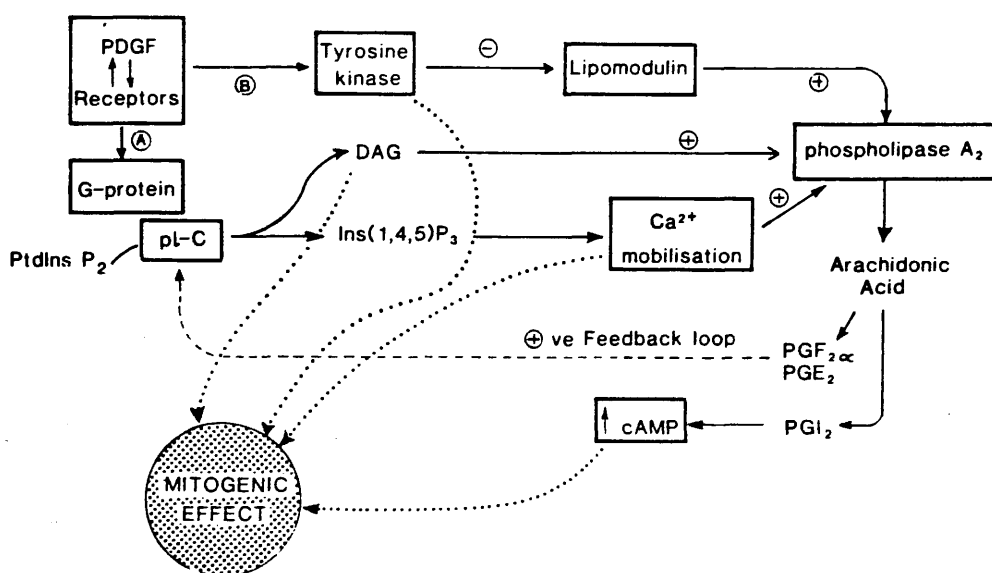


Figure 5: Potential mechanisms by which platelet derived growth factor can influence inositol lipid metabolism and mitogenesis

rise in intracellular free Ca^{2+} might itself lead to mitogenesis or, along with the rise in DG, could activate PLA_2 and result in the formation of free AA. As before, this may be metabolised by cyclooxygenase and other enzymes to yield PGE_2 and $\text{PGF}_{2\alpha}$ and to PGI_2 , but the formation of PGE_2 and $\text{PGF}_{2\alpha}$ here, would act to amplify the action of phospholipase C.

2. MATERIALS AND METHODS

2. Materials and Methods

2.1 Methods of cell culture

Swiss mouse 3T3 fibroblasts can be grown as monolayers on either plastic or glass surfaces. They can also be grown in roller bottles in combination with microcarrier beads.

In this study, the methods of cell culture were essentially those used by Dr L. Jimenez de Asua (personal commun.).

Swiss 3T3 cells were grown in monolayer culture at 37°C in an automatic CO₂ incubator (Forma Scientific, Marietta, Ohio, U.S.A.) under a humidified atmosphere of 95% air/5% CO₂ in Dulbecco's Modification of Eagle's Medium (DMEM). The medium was supplemented with foetal calf serum (6.2%), new born calf serum (1%), benzyl penicillin (80 Iu/ml) and streptomycin (80 µg/ml).

2.1.1 Maintenance of aseptic conditions

All aseptic manipulations were performed in a vertical laminar flow hood (Microflow Ltd., Andover, Hants., U.K.). Every operation utilising sterile solutions included the use of either 1 ml sterile graduated pastettes (Alpha Labs., Eastleigh, England) or 10 ml sterile graduated plastic pipettes (Falcon), when necessary. Operations with 10 ml pipettes were made easier by the use of an automatic 'Pipetus' obtained from Flow Labs., Irvine, Scotland. When solutions were to be sterilized either 0.2 micron Nalgene filter units (Nalge Company, Rochester, U.S.A.) for volumes over 50 ml, or 0.2 micron microflow 25 filters (Flow Labs.) were used, depending on the filtration volume.

2.1.2 Passaging of Swiss 3T3 cells

Cells were seeded at a density of approximately 5×10^4 per 60 mm plastic petri dish or approximately 2×10^4 cells per 20 mm plastic well (6-well multidish), in 6 ml or 2 ml culture medium respectively. On the day of passaging, the appropriate number of confluent dishes were taken to give the required seeding density of the new passage. Normally a split ratio of 1:10 was used, and this yielded the cell plating numbers shown above.

Firstly, the 'spent' medium from the confluent petri-dishes was aspirated off using an autoclaved pasteur pipette attached to a suction line. 1 ml of a trypsin-EDTA solution (0.05% trypsin 1:250 and 0.02% EDTA in a special salt solution; Flow Labs.) was added to each dish, which was then gently shaken back and forth. The trypsin-EDTA was aspirated off and another 1 ml was added to the dishes. The process was then repeated. Finally, 0.2 ml trypsin-EDTA was added to each dish. Again the dishes were gently shaken and then placed in the incubator at 37°C for 3-4 minutes. After this time, the dishes were inspected under an inverted microscope to check that the cells were floating as single cells. 6 ml fresh serum-containing medium was next added to each dish (this inactivated the trypsin-EDTA solution) and this and the floating cells were then accumulated in a 250 ml flask (Nunc). Fresh medium was added to the flask to give a cell concentration of approximately 9×10^3 cells/ml medium. A haemocytometer was used to check the Swiss 3T3 cell concentration

of this final suspension. 6 ml or 2 ml (for dish or well) aliquots were then added to new petri-dishes or multiwells.

2.1.3 Feeding of Swiss 3T3 cells

3-4 days after plating, the cells were fed. The cells were then left for one week to allow them both to become confluent and to round up and form the characteristic "cobblestone" appearance.

The process of feeding the Swiss 3T3 cells was relatively simple. 'Spent' medium was aspirated as described above and 6 ml/2 ml fresh medium was then added to each dish/well. The petri-dishes/multiwells were then placed in the incubator.

It should be pointed out that one of the problems which can arise when Swiss 3T3 cells are grown is that the cells can spontaneously transform. This can be detected by observing the petri-dishes under a microscope. One can observe "foci" which are small areas of the dish where cells have transformed. The foci appear as small white spots as the cells are densely packed together and are no longer growing in a monolayer.

The appearance of transformed foci in the petri-dishes renders the passage of cells useless and, upon detection, a fresh batch of cells would be thawed from liquid N₂ storage and grown for further experiments (see later). The experimental "lifetime" of each batch of cells is therefore rather short; about 3-4 months.

Another source of variability experienced in growing Swiss 3T3 cells to confluence is that the more passages cells are given, the faster they grow. In some cases, cells were

passaged and not fed, before being used in experiments seven days after plating. Fortunately, this did not appear to affect the results of the experiments, in that $\text{PGF}_{2\alpha}$ induced a similar increase in, for example, InsP formation, in both 'fed' and 'unfed' cells.

2.1.4 Storage of Swiss 3T3 cells in liquid nitrogen

Confluent dishes were taken and the Swiss 3T3 monolayers trypsinized in the usual manner. Cells were resuspended in fresh growth medium at a concentration of $3-5 \times 10^6$ cells/ml. The cell suspension was cooled in an ice-water bath prior to the addition of glycerol to give a final concentration of 10% (v/v). 1 ml aliquots of the cell suspension were transferred to sterile plastic ampoules (Flow Labs.) using a syringe with an 18G needle. The ampoules were heat-sealed before being attached to metal straws, which were then placed in polystyrene boxes and put into a -70°C freezer for approximately 2 hours. It has been shown that this procedure gives a cooling rate of approximately 1°C min^{-1} . The straws were then placed in a canister in the vapour phase of a liquid nitrogen (N_2) container (Union Carbide, Darlington, U.K.).

When frozen Swiss 3T3 cells were to be recovered, an ampoule was carefully removed from the liquid N_2 container and placed into a 37°C water bath for thawing. The ampoule was then washed with 70% ethanol and allowed to dry at room temperature. The end of the ampoule was broken off and the cell suspension aseptically transferred to a 90 mm petri-dish (Nunc), using a sterile syringe. 9 mls of fresh serum-

containing medium was added and the cells placed into the incubator at 37°C. The cells were grown until they were almost confluent (with medium changes every 3-4 days). Following this growth period the "new" Swiss 3T3 cells were used for passaging.

2.2 Dialysis of serum

Newborn calf serum (Flow Labs.) was dialysed at 4°C to remove, amongst other things, growth factors of a molecular weight less than 3,500, e.g. $\text{PGF}_{2\alpha}$, vasopressin and gastrin-releasing peptide (GRP - a bombesin-like growth factor) using Micropor dialysis tubing (Spectra/Por 3, Pierce and Warriner, London), thereby leaving growth factors like PDGF and EGF remaining.

Tubing (about 25 cm long) was washed with filtered distilled water, one end was tied and the tubing then filled with 20 ml newborn calf serum (under sterile conditions). The other end was then tied, and the tubing placed in a beaker containing 5 litres 0.9% (w/v) NaCl. The beaker was stored at 4°C for 4 days, with the dialysing solution changed daily. The dialysed serum was then transferred to sterile tubes (Nunc) and stored in 5 ml aliquots. A second sample of newborn calf serum from the same batch was subjected to similar conditions of temperature etc., but was not dialysed. This non-dialysed serum was used as a control in experiments in which the effects of dialysed serum was compared to those of non-dialysed serum.

Previous work has shown that sera prepared in this way has unimpaired ability to stimulate cell proliferation (Besterman et al., 1986).

2.3 The radioactive precursors used

In this project, three precursors were used: [$2\text{-}^3\text{H}$] glycerol (40 $\mu\text{Ci}/6$ ml petri-dish) was used to allow the measurement of the cellular content of the major phospholipids i.e., phosphatidylinositol (PtdIns), phosphatidic acid (PtdA), phosphatidylcholine (PtdCho), phosphatidylserine (PtdS) and phosphatidylethanolamine (PtdE). In such cases, dishes of Swiss 3T3 cells were pre-labelled for 2 days with the isotopically-labelled glycerol.

Myo- [$2\text{-}^3\text{H}$] -inositol (5 $\mu\text{Ci}/6$ ml dish or 2 ml well), was used to allow the measurement both of the inositol-containing phospholipids: PtdIns, phosphatidylinositol 4-phosphate (PtdIns 4P) and phosphatidylinositol (4,5)-bisphosphate (PtdIns(4,5)- P_2) and also the measurement of cytoplasmic inositol-containing metabolites: inositol, inositol monophosphate (InsP), inositol bisphosphate (Ins P_2), inositol trisphosphate (Ins P_3) and inositol tetrakisphosphate (Ins P_4). Again, cells were pre-labelled for 2 days with [^3H] -inositol prior to the experiment.

[5,6,8,9,11,12,14,15- ^3H] -Arachidonic acid (AA), (0.5 $\mu\text{Ci}/6$ ml dish or 2 ml well) was used to allow the measurement of free AA and prostaglandins in the medium bathing the cells. Radiolabelled [^3H] -arachidonyl PtdA was also measured with this protocol, to show any effects

on the turnover of the inositol lipid cycle. Cells were pre-labelled for 20-24 hours with [^3H] -AA prior to the experiment.

Pre-labelling the Swiss 3T3 cells with the desired radioactive precursor, for these relatively long periods, leads to an isotopic equilibrium being attained, in this case changes in, for example the radioactivity associated with a particular phospholipid, were taken as reflecting changes in the absolute cellular content of that lipid (Drummond, A.H., unpublished results).

The basic protocol for each experiment was as follows: the medium from the radiolabelled cells was aspirated and discarded (except for experiments in which cells were pre-labelled with [^3H] -AA). The cells were then washed twice with 6 ml/dish or 2 ml/well of a Hepes-buffered balanced salt solution (BSS; see Table 3 for constituents) at 37°C. and left in a final volume of 6 ml/2 ml BSS per dish/well. The dishes were returned to the incubator and left for 10 min, so that the cells would equilibrate at the reaction temperature. Thereafter, drugs were added to the dishes in various concentrations and combinations and for various lengths of time. The reactions were stopped by removing each dish from the incubator at the desired time. The BSS was quickly aspirated and discarded (or kept, in the case of experiments involving the measurement of [^3H] -AA release into the bathing medium). One ml ice-cold 10% (w/v) trichloroacetic acid (TCA) was then added immediately to the dish which was thereafter placed on an ice-bath until all of the samples had been collected.

Table 3: Constituents of balanced salt solution.

135 mM	NaCl
4.5 mM	KCl
1.5 mM	CaCl ₂
0.5 mM	MgCl ₂
5.6 mM	glucose
10 mM	HEPES
0.1% (w/v)	bovine serum albumin
	pH 7.4

N.B. 10 mM NaCl was replaced with 10 mM LiCl where indicated in the text.

2.4 Phospholipid extraction

After cell activation had been stopped for all of the dishes, cells from each dish/well were scraped, into test-tubes, using a "rubber policeman". These were centrifuged (3000g; 10 min; 4°C). The supernatant was removed and kept for inositol phosphate analysis (if the radiolabel used was [³H]-inositol). The cell pellet was then extracted essentially by the method of Schacht et al. (1981). 1.5 ml chloroform: methanol (1:2 v/v), 0.5 ml 2.4N HCl 0.5 ml distilled H₂O and 0.5 ml chloroform was added to the test-tubes (A). After 20 min, (to allow the lipids to be extracted into the organic phase), the samples were centrifuged (3000g; 5 min; 20°C). The lower phase (organic phase) was transferred to other test-tubes (B), while the upper phase was further extracted with 1 ml chloroform. After a further centrifugation step to break the two phases, the lower phase was once again added to the test-tubes (B). Finally 2 ml 1N HCl: Methanol (1:1 v/v) was added to test-tubes (B) and these were centrifuged as above. The upper phase was discarded and the lower phase transferred to glass vials. The lower phase was then evaporated to dryness at 40°C under oxygen-free nitrogen, using a sample concentrator (Techne Ltd., Cambridge, England) and the residue redissolved in 100 µl chloroform: methanol (9:1 v/v) and stored at -20°C until phospholipid separation was carried out. Generally, phospholipids were separated within 48 hours of extraction. The above method was used in all cases where phospholipids were extracted,

whether the cells were radiolabelled with [^3H] -glycerol, [^3H] -inositol or [^3H] -AA. The one exception to this, was when very short time course experiments were carried out to study the effects of $\text{PGF}_{2\alpha}$ on the inositol-containing phospholipids. In this case, the method used was essentially that of Creba et al. (1983). To the cell pellet was added 2 ml dist. H_2O and 3.75 ml chloroform:methanol: 12M HCl (50:100:1). After 20 min (to allow extraction of the lipids), samples were centrifuged at 3000g for 5 min (20°C). The upper phase was discarded and replaced by 2 ml of the synthetic upper phase, obtained from a mixture of chloroform:methanol: 1M HCl (1:1:0.9). After thorough vortexing and centrifugation 1 ml of the lower (organic) phase was taken and deacylated for 20 min at room temperature (20°C) with methanolic NaOH (0.2 ml methanol plus 0.2 ml of 1M NaOH in methanol: H_2O (19:1)). After this time, 1 ml chloroform, 0.6 ml methanol and 0.6 ml distilled H_2O were added and the samples were mixed and centrifuged to separate the phases. The upper phase (now containing the labelled deacylated inositol lipids) was diluted to 5 ml to give a final concentration of 5 mM $\text{Na}_2\text{B}_4\text{O}_7$ before separating the deacylated lipids on Dowex anion exchange columns (see next section).

2.4.1 Phospholipid separation

For cells labelled with [^3H] -glycerol and [^3H] -AA, phospholipids were separated by a rapid two dimensional thin-layer chromatography (TLC) method on silica gel G plates (0.25 mm; 10 x 10 cm, Polygram; Camlab, Cambridge, England)

as described by Yavin and Zutra (1977), but with minor modifications. The following mixtures were prepared in a final volume of 200 ml; chloroform:methanol: 40% (v/v) methylamine (13:6:1.5, by volume) for the first direction; diethyl ether: glacial acetic acid (19:1, by volume) for the intermediary run (though in the second direction); and chloroform : acetone : methanol : acetic acid : dist. H₂O (10:4:2:3:1, by volume) for the third run (second dimension). The chromatographic chambers (Multitank, Camlab) were lined at both ends with Whatman filter paper, and the solvent mixtures added about 1-2 hours before samples were run. All three organic mixtures were freshly prepared and at least 30 plates could be run with a single batch of solvents, without loss of resolution. Phospholipid samples were applied using 10 µl disposable micro-pipettes (Camlab), to the lower left hand corner (2 cm from the edges) on a point lightly marked with a pencil. The solvent from each sample was evaporated in a stream of warm air from a blow dryer placed about 40 cm from each spot. Each glass vial (containing phospholipid sample) was washed with a further 30 µl of chloroform: methanol (9:1 v/v) which was also spotted as described above. Carrier phosphatidate (5 µg) was used to aid visualisation of the PtdA spot in this system, since the cellular content of this lipid in Swiss 3T3 cells was rather low and only occasionally visible under iodine staining. The chromatoplates, eight at a time, were placed in the first solvent. Plates were developed for approximately 20 min until the solvent front was 1 cm from the edge. Plates were

removed and placed on a tray for drying. This was performed with warm air from 3 blow dryers, located around the tray, for a period of 10-15 min. A thorough dry at this stage was essential to enable complete neutralisation of the base (methylamine) at the following stage. The silica gel layer was then exposed to the fumes of a concentrated HCl (10.8M) solution for 5 min, the distance between the exposed gel and HCl surface being about 2.5 cm. The plates were then dried with blow dryers, as above, in warm and ambient air for 5 min and 3 min respectively. They were then placed for about 15 min into the second chromatography chamber, the origin now at the lower right corner, to eliminate impurities, which would otherwise interfere with the subsequent second dimension elution. Following this ether/acetic acid wash, the plates were dried for 5 min at ambient temperature and were rerun in the same direction for about 25 min in the third chromatography chamber, containing the chloroform : acetone : methanol : acetic acid : dist. H₂O mixture. After thorough drying, the phospholipid spots (PtdA, PtdIns, PtdS, PtdCho, PtdE) were identified by comparison with reference standards using iodine vapour.

For cells labelled with [³H] -inositol, the phosphoinositides (PtdIns, PtdInsP and PtdInsP₂) were extracted by the method already described, except in the short-time course experiments where deacylated lipids were extracted by the method of Creba et al. (1983), also described above.

Generally, the phosphoinositides were separated on oxalate-impregnated HPTLC plates (20 cm x 10 cm, Merck) following the procedure of Jolles et al. (1981).

It was found that an unknown phospholipid co-migrated with PtdIns⁴-P in this system. This was thought to be lyso PtdIns⁴-P, a phospholipid which is not present in GH₃ pituitary cells, to any significant extent (Drummond, A.H., unpublished results). Due to the difficulty in separating the polyphosphoinositides from one 'unknown' phospholipid, a different method of separating them was used in the short-time course experiments with PGF_{2α}, where deacylated lipids were separated essentially by the method of Creba et al. (1983).

Samples (deacylated lipids) were placed onto columns (1 x 2 cm) of Dowex (1×8 100-200 mesh, Cl⁻ form) anion exchange resin, which had been washed with 2.4M ammonium formate to change the resin to a formate form. 10 ml of 5 mM Na₂B₄O₇/0.18M NH₄COOH was used to elute glycerylphosphorylinositol (GroPIns), 10 ml 0.1M formic acid/0.3M NH₄COOH for glycerylphosphorylinositol monophosphate (GroPInsP) and 10 ml 0.1M formic acid/0.75M NH₄COOH for glycerylphosphorylinositol bis-phosphate (GroPInsP₂).

2.5 Isolation of [³H] -inositol phosphates

As already described above, agonist-induced cell stimulation was stopped by the addition of 1 ml ice-cold 10% (w/v) TCA. The cells were then scraped off the petri-dishes into test-tubes and centrifuged (3000g; 10 min; 4°C). The resulting

supernatant (the acid-soluble fraction) was collected and frozen at -20°C until $[\text{}^3\text{H}]$ -inositol phosphates were to be assayed. At this stage, the pellet for inositol-containing lipids was extracted as described above.

For the separation of acid-soluble $[\text{}^3\text{H}]$ -inositol metabolites, frozen aliquots were thawed and extracted three times with three volumes of water-saturated diethyl ether. After driving off the residual ether in a boiling water bath, the extracts were allowed to cool at room temperature. The extracts were then placed on to columns (1 x 2 cm) of Dowex anion exchange resin (treated as described above). The method used, thereafter, was similar to that described by Downes et al. (1986). $[\text{}^3\text{H}]$ -Inositol was initially eluted with 7 ml distilled water, but due to the extremely high levels of $[\text{}^3\text{H}]$ -inositol found in Swiss 3T3 cells, was subsequently eluted with 24 ml dist. H_2O . $[\text{}^3\text{H}]$ -InsP was eluted with 8 ml 0.1M formic acid/0.2M NH_4COOH ; $[\text{}^3\text{H}]$ -InsP₂ was eluted with 8 ml 0.1M formic acid/0.4M NH_4COOH ; $[\text{}^3\text{H}]$ -InsP₃ was eluted with 8 ml 0.1M formic acid/0.8M NH_4COOH and $[\text{}^3\text{H}]$ -InsP₄ was eluted with 8 ml 0.1M formic acid/1.2M NH_4COOH . Unfortunately, the radioactivity measured for the InsP₄ elution was similar to background, under the conditions of these experiments, and hence drug effects on InsP₄ levels could not be determined.

2.5.1 High performance liquid chromatographical analysis of the inositol phosphates

In order to detect the nature of the inositol phosphate isomers present in resting and agonist-stimulated Swiss 3T3

cells, HPLC analysis of the acid soluble fractions were carried out.

Neutralised samples (0.5 ml) were injected on to a Partisil 10SAX analytical HPLC column, (25 cm x 0.4 mm), preceded by a guard column of similar material (5 cm x 0.4 mm) (Hichrom, Reading, England), and the column washed with deionised, degassed, ultrapure H₂O for 30 min at a flow rate of 1.25 ml/min. This prolonged H₂O elution was found necessary to eliminate the extremely high levels of [³H] -inositol found in Swiss 3T3 cells. Thereafter, a linear gradient of up to 2M NH₄COOH, buffered to pH 3.7 with orthophosphoric acid (22°C) was applied over the subsequent 40 min period. Fractions were collected every 20s and counted by liquid scintillation spectrometry after addition of 3 ml Optiphase-Safe (LKB, FSA Labs, Loughborough, England). The column was calibrated with a series of standards both before and after running the 3T3 cell extracts.

2.6 Isolation of [³H] -AA metabolites

In experiments where [³H] -AA-derived metabolites were to be measured, at the end of drug incubations, the 6 ml BSS/dish (2 ml/well) surrounding the cells was removed into test tubes and kept on ice. The cells then received 1 ml ice-cold 10% (w/v) TCA and were scraped off the petri-dishes into test-tubes and centrifuged, as previously described.

A fraction of the BSS (0.5 ml) was then placed directly in 5 ml Ecoscint and its radioactivity (cpm or dpm) was measured by liquid scintillation spectrometry. This gave

a measure of [^3H] -AA-derived radioactivity released from the cells.

The remaining BSS was, on a number of occasions extracted for radiolabelled AA and more polar metabolites such as prostaglandins, essentially by the method of Ubatuba (1978). The BSS was acidified with concentrated HCl to pH 3. The acidified samples were then extracted twice with two volumes of cyclohexane : ethylacetate (1:1 v/v) and the upper phase collected each time. The combined upper phases were then dried under oxygen-free nitrogen (at 40°C) and reconstituted in absolute ethanol (100 μl).

The labelled [^3H] -AA metabolites were then separated by TLC using the SMI solvent system as described by Ubatuba (1978): ethylacetate : acetone : acetic acid (90:10:1, by volume). This system was designed to allow greater separation between 6 keto PGF $_{1\alpha}$ and PGE $_2$ than other solvent systems detailed by Ubatuba.

Metabolites were identified by comparison with reference standards, and visualised as described below. The TLC plates (20 cm x 20 cm, Merck) were marked lightly with a pencil and each sample was spotted on to 1 cm bands of the plates. The origin was 2 cm from the bottom of the plates. The plates were placed in the chromatography chamber containing the solvent for about 25-30 min (until the solvent front was at least 2/3 way up the plates). The plates were then removed and the solvent evaporated at room temperature in a fume cupboard. The plates were sprayed in the fume cupboard with a reagent containing absolute ethanol: conc. H $_2$ SO $_4$:

anisaldehyde (10:1:1, by volume). The 6 keto $\text{PGF}_{1\alpha}$ appeared as a bright yellow band, at this stage, and it was marked lightly with a pencil for each sample. The plates were then placed in an oven and heated for 10 min at approximately $80-90^{\circ}\text{C}$ until the other coloured bands appeared: PGD_2 , orange/brown; PGE_2 , brown; $\text{PGF}_{2\alpha}$ and AA, dark blue/violet and TxB_2 , grey/blue.

2.7 Determination of radioactivity

After the iodine had sublimed, identified lipid spots/bands were scraped, using a surgical blade, and incorporated radioactivity quantified by liquid scintillation spectrometry (Packard, model 3390 or Packard model 2000 CA). 1 ml aliquots of the separated $[^3\text{H}]$ -glycerylphosphorylinositol phosphates were quantified in a similar manner, as were 1 ml aliquots of the separated $[^3\text{H}]$ -inositol phosphates. The scintillation fluid generally used was Ecoscint (National Diagnostics, Aylesbury) 5 volumes/volume sample. Optiphase-Safe (LKB from FSA Labs, Loughborough) was used in experiments where HPLC analysis was carried out on the inositol phosphates.

2.8 Protein determination for Swiss 3T3 cells

Cellular protein content was determined by the method of Lowry et al. (1951) using bovine serum albumin (Sigma) as standard. Petri-dishes (6 ml) or multiwells (2 ml) of 3T3 cells were scraped into test-tubes and centrifuged at $3000g$ for 5 min at room temperature. The pellet was washed twice with 0.9% (w/v) saline, undergoing vortexing and centrifugation each time ($3000g/3$ min). This removed any serum albumin present.

From random samples the cellular protein content of 3T3 cells/dish or well was as follows: 0.48 ± 0.045 mg protein/10 cm diameter petri-dish; 0.17 ± 0.01 mg protein/6 cm diameter petri-dish and 0.05 ± 0.005 mg/2 ml diameter well.

2.9 Statistical presentation of results

Results are expressed as the mean \pm standard error of the mean. Data, where indicated, were subjected to a Student's t-test to determine whether a significant difference existed at the 5% level between the mean values. Levels of significance used throughout, unless otherwise stated:

* $p < 0.05$, ** $p < 0.001$.

2.10 Sources of chemicals used

The various drugs, culture materials and biochemical compounds used in addition to standard laboratory reagents are listed in Table 4, together with their sources. All solutions were prepared with glass distilled H_2O unless otherwise stated.

Table 4: Chemicals and cell culture materials used and
their sources

Material	Source
Swiss 3T3 cells	Kindly donated by the following people: Dr K.D. Brown (Cambridge, England); Dr G. Thomas (Basel, Switzerland) and Dr L. Jimenez de Asua (London, England)
Petri-dishes, flasks, multiwells, filters etc.	Flow Labs., Irvine, Scotland or Gibco-Europe, Paisley, Scotland
Dulbecco's Modification of Eagle's Medium (DMEM)	Flow Labs
Foetal bovine serum	Flow Labs
Newborn bovine serum	Flow Labs
L-Glutamine (200 mM)	Flow Labs
Trypsin-EDTA solution	Flow Labs
Penicillin (500 I.U./ml)/ Streptomycin (500 µg/ml)	Flow Labs
Trypan Blue solution	Flow Labs
Micropor (Spectrapor 3) dialysis tubing	Pierce and Warriner, London, England
myo-[2- ³ H] -inositol (16.4/mmol)	Amersham International p.l.c., Amersham, England.
[2- ³ H] -glycerol (1 Ci/mmol)	Amersham International p.l.c.
[5,6,8,9,11,12,14,15- ³ H] - AA (135 Ci/mmol)	Amersham International p.l.c.
Dowex 1 x 8 (Cl ⁻ form, 100-200 mesh) resin	Sigma Chemical Co. Ltd., Poole, England.
Partisil 10 SAX HPLC column	Hichrom, Reading, England
Optiphase-Safe	(LKB) F.A.S. Labs, Loughborough, England
Ecoscint	National Diagnostics, Aylesbury, England

Table 4 (Contd).

Material	Source
HEPES	Sigma Chemical Co. Ltd.
Folin and Ciocaltean's phenol	BDH Chemicals Ltd., Poole, England
Lithium chloride	BDH Chemicals Ltd.
Bovine serum albumin (99% essentially fatty acid free)	Sigma Chemical Co. Ltd.
$[^3\text{H}]$ -Ins1P $[^3\text{H}]$ -Ins4P	Amersham International p.l.c. Was prepared by alkaline hydrolysis of $[^3\text{H}]$ -PtdInsP (Amersham) by the method of Grado and Ballou (1961)
$[^3\text{H}]$ -Ins(1,4) P_2 $[^3\text{H}]$ -Ins(4,5) P_2	Amersham International p.l.c. Was prepared by alkaline phosphatase action on $[^3\text{H}]$ - Ins(1,4,5) P_3 (Amersham) (Hughes & Drummond, 1987)
$[^3\text{H}]$ -Ins(1,4,5) P_3	Amersham International p.l.c.
Prostaglandin $\text{F}_{2\alpha}$	Sigma Chemical Co. Ltd.
Prostaglandin standards	Sigma Chemical Co. Ltd.
Arachidonic acid	Sigma Chemical Co. Ltd.
AA analogues	Sigma Chemical Co. Ltd.
Lipid standards	Sigma Chemical Co. Ltd.
Melittin	Sigma Chemical Co. Ltd.
Dexamethasone	Sigma Chemical Co. Ltd.
Indomethacin	Sigma Chemical Co. Ltd.
Flurbiprofen } H_2O soluble BW755C } form	Kindly donated by Dr D.E. MacIntyre, Merck Labs, New York, U.S.A.

Table 4 (Contd).

Material	Source
PDGF - highly purified form	Kindly donated by Dr C.-H. Heldin, Uppsala, Sweden

3. RESULTS

3. Results

3.1 The effects of prostaglandin $F_{2\alpha}$ on inositol lipid metabolism in Swiss 3T3 cells

Research prior to this project had shown that $PGF_{2\alpha}$, when added to quiescent Swiss 3T3 cells, not only led to a stimulation of DNA synthesis, but also stimulated the incorporation of $[^{32}P] -PO_4$ into PtdIns. It had also been observed that $PGF_{2\alpha}$ caused a small increase in the cellular content of DG (Macphée et al., 1984). However, at that time, little detailed work had been done to investigate the effects of $PGF_{2\alpha}$ on the inositol lipid cycle in 3T3 cells. Therefore, the first section of this project was concerned with studying the effects of $PGF_{2\alpha}$ on the inositol-containing phospholipids (phosphoinositides), the inositol phosphates and other membrane phospholipids.

3.1.1 Studies using Swiss 3T3 cells pre-labelled with $[^3H] -inositol$

3.1.1.1 Amplifying responses using lithium chloride

In the following experiments, 10 mM lithium chloride was used to amplify receptor -stimulated responses (see Methods). The effect of using Li^+ ions can be seen clearly in Figure 6. Here, the effects of $PGF_{2\alpha}$ on the inositol phosphates are compared for cells bathed in normal (full Na^+) balanced salt solution (BSS) and cells bathed in Li^+ -containing BSS. It is apparent, that Li^+ amplifies the increase in InsP levels, induced by $PGF_{2\alpha}$.

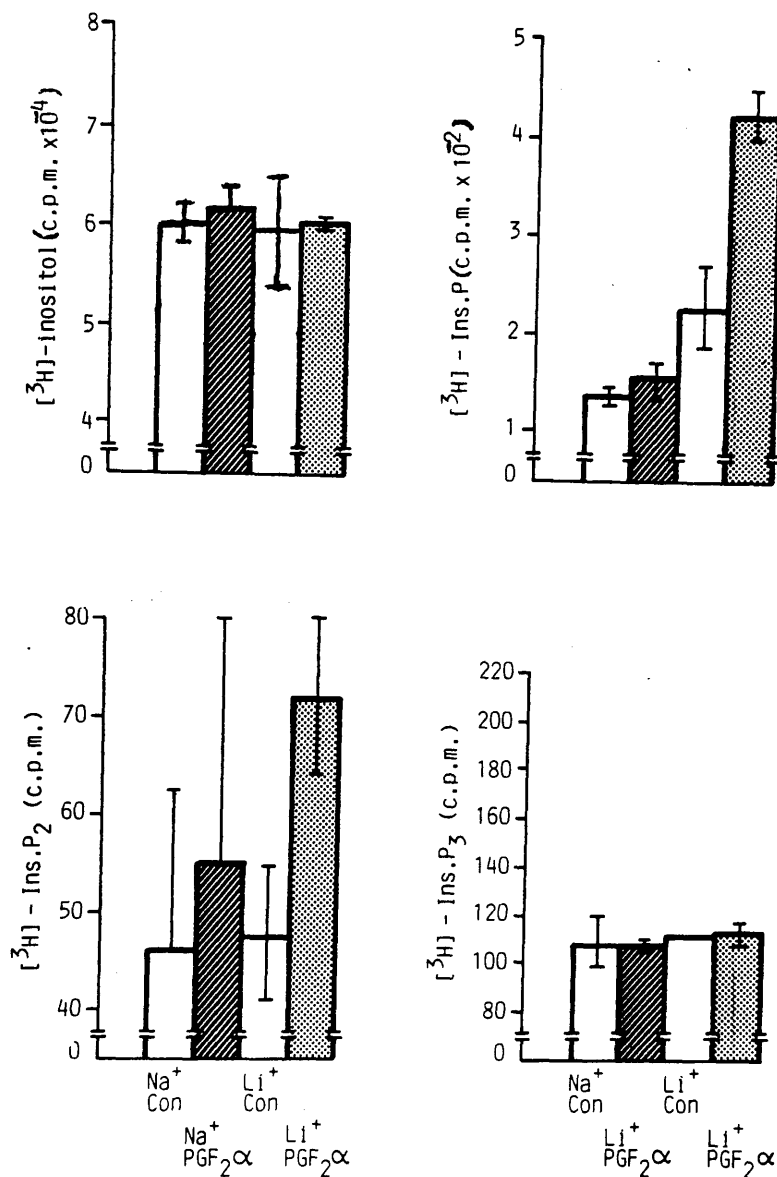


Figure 6: The effect of 10 mM LiCl on acid soluble [³H] - inositol phosphate levels in PGF₂α-stimulated Swiss 3T3 cells

Swiss 3T3 cells were labelled to equilibrium with 5 μ Ci [³H] -inositol/dish, (see Methods). They were washed and left in BSS which either had a full Na⁺-complement or contained 10 mM Li⁺ in place of 10 mM Na⁺. PGF₂α (1.6 μ M) was used as an agonist and the reactions were terminated after 10 min. Acid-soluble [³H] -inositol-derived radioactivity was quantified as described in the Methods section. The values shown are from a representative experiment conducted in duplicate and repeated once.

This effect is due to lithium's ability to block the monophosphatase enzymes, which are involved in the hydrolysis of InsP to inositol (Figure 2). However, lithium is also thought to affect other enzymes in the inositol lipid cycle and this has been discussed in Section 1.1.4.

Lithium had no significant effect on cellular inositol levels, and this suggests that Swiss 3T3 cells contain a very large pool of inositol, which is difficult to deplete. Care is necessary in separating Ins from InsP on Dowex columns, because of the marked disparity in their cellular contents and thus in later experiments 24 ml distilled H₂O was used to elute the inositol fraction.

Lithium had no significant effect on InsP₂ and InsP₃ accumulation. However, if there was any trend to be seen in these results it would be that lithium slightly amplified the effects on InsP₂ levels in response to PGF_{2α}.

It is noteworthy that the radioactivity, in c.p.m., recorded for InsP₂ and InsP₃ is extremely low and this made it difficult to measure accurately any changes due to agonists.

In all subsequent experiments, unless otherwise stated, 10 mM lithium chloride was used to amplify the response to agonists, measured as a change in the level of InsP.

3.1.1.2 The dose-response relationship for PGF_{2α}-induced changes in inositol lipid metabolism

Before the effects of PGF_{2α} on the various metabolites of the inositol lipid cycle were studied in detail, a concentration-response curve for PGF_{2α} was constructed, in

which the increase in the level of InsP was taken as a measure of cycle stimulation. Therefore, 3T3 cells were incubated for 10 min with concentrations of $\text{PGF}_{2\alpha}$ ranging from 1 nM to 10 μM (Figure 7). The maximum response was obtained using 1 μM $\text{PGF}_{2\alpha}$ and half-maximal stimulation at approximately 30 nM. In the following experiments, unless otherwise stated, a supramaximal concentration of $\text{PGF}_{2\alpha}$ (1.6 μM /500 ng/ml) was used.

3.1.1.3 The effect of $\text{PGF}_{2\alpha}$ on the 3T3 cell content of the inositol-containing phospholipids

As before, 10 mM lithium chloride was used in these studies. Figure 8 shows the time course of the effects of $\text{PGF}_{2\alpha}$ on cellular polyphosphoinositide levels. It must be pointed out that Figure 8 shows the results of one representative experiment of three, and in this case control samples were only taken at 0 and 5 minutes. However, in the other experiments control samples were taken throughout the 20 minutes duration of the drug incubation, and the results from these experiments (not shown) indicated that levels of $\text{PtdInsP} + \text{PtdInsP}_2$ did not change significantly over this time.

The combined levels of PtdInsP and PtdInsP_2 in 3T3 cells rose to a maximum within 40 seconds of administration of $\text{PGF}_{2\alpha}$. Levels of the polyphosphoinositides remained elevated above basal over the 20 minutes duration of the drug incubation.

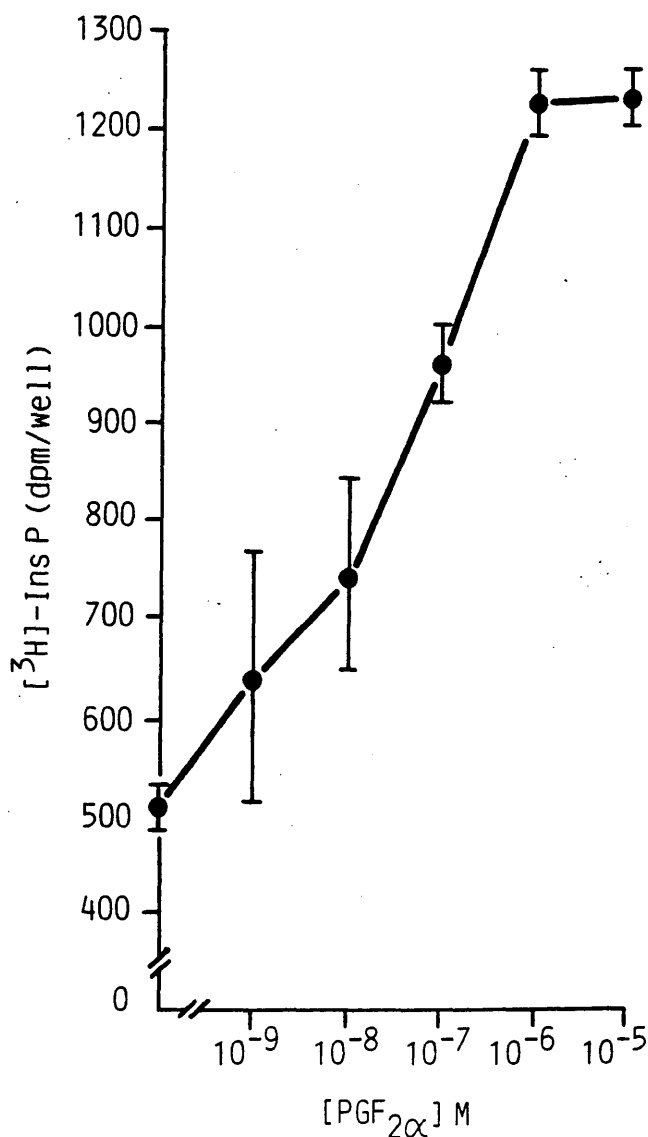


Figure 7: The effect of increasing concentrations of PGF_{2α} on [³H]-InsP levels in Swiss 3T3 cells

Cells were labelled to equilibrium with 5 μ Ci [³H]-inositol/well. They were washed and left in BSS containing 10 mM LiCl, and incubated for 10 min with PGF_{2α}. For experimental details see Methods section. The data shown are from one experiment conducted in triplicate and repeated once with similar results.

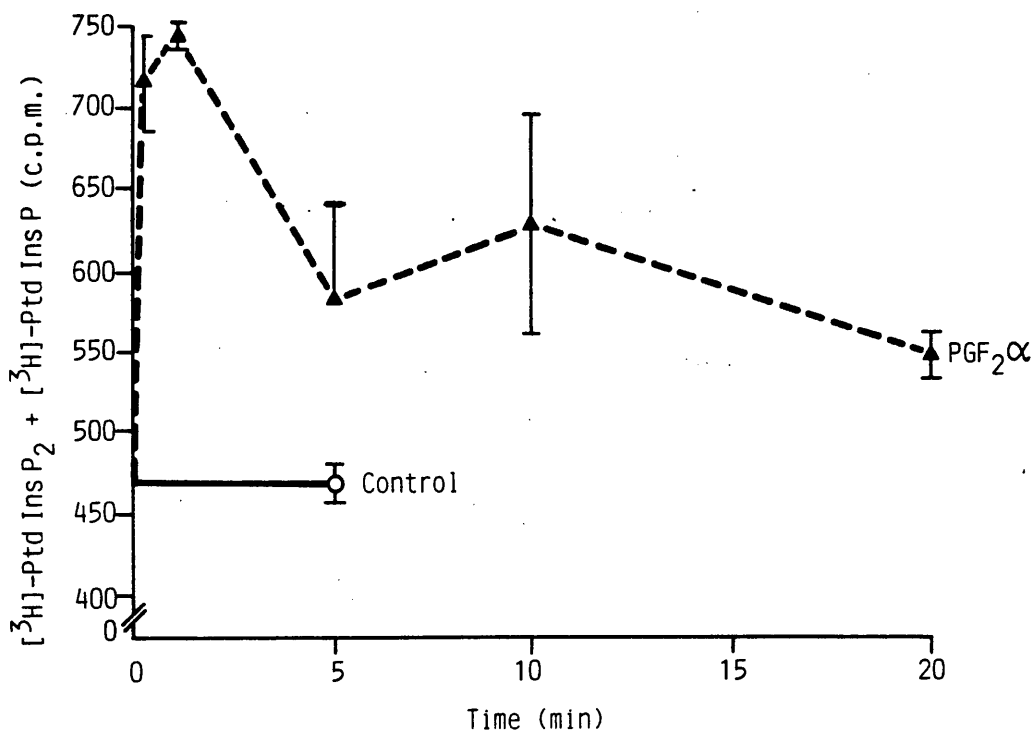


Figure 8: The time course of the effects of PGF₂α on the polyphosphoinositide content of Swiss 3T3 cells

Cells were labelled to equilibrium with 5 μ Ci [3 H] -inositol/dish. They were washed and left in BSS containing 10 mM LiCl, and incubated with 1.6 μ M PGF₂α. For experimental details see Methods section. The values shown are from a representative experiment conducted in duplicate and replicated twice.

However by analogy with the response of other cell types (e.g. GH₃ pituitary cells) to hormones it was expected that there would be an initial decrease in PtdIns and PtdInsP₂ levels occurring before resynthesis of the lipids can take place (Macphee and Drummond, 1984). In contrast, a rather rapid rise in the polyphospho-inositide levels was noted. Thus it seemed important to repeat this series of experiments using a protocol in which earlier time points were measured.

In these experiments, a different method of analysing the phosphoinositides was used, which involved the deacylation of the extracted lipid and separation of glyceryl phospho-inositol phosphates on Dowex anion exchange columns (see Methods - Creba et al., 1983).

The results shown in Figure 9.a indicate that as before PtdInsP levels rose after 40s of administration with PGF_{2α}. However, here the results show that a slight decrease in PtdInsP content occurred within the first 10s of stimulation.

Levels of PtdInsP₂ (Figure 9), again, showed only an increase that reached a peak after 40s. Unlike levels of PtdInsP, there was no significant decrease in the level of PtdInsP₂ within the first 10s of the stimulation. The levels of PtdIns, measured by either method, did not significantly increase in response to PGF_{2α} (Figure 9b).

3.1.1.4 The effect of PGF_{2α} on the 3T3 cell content of the acid-soluble inositol phosphates

The ability of PGF_{2α} to alter the cellular content of the acid-soluble metabolites of PtdInsP₂, namely, inositol, InsP, InsP₂, InsP₃ and InsP₄, was investigated (Figures 10, 11, 12).

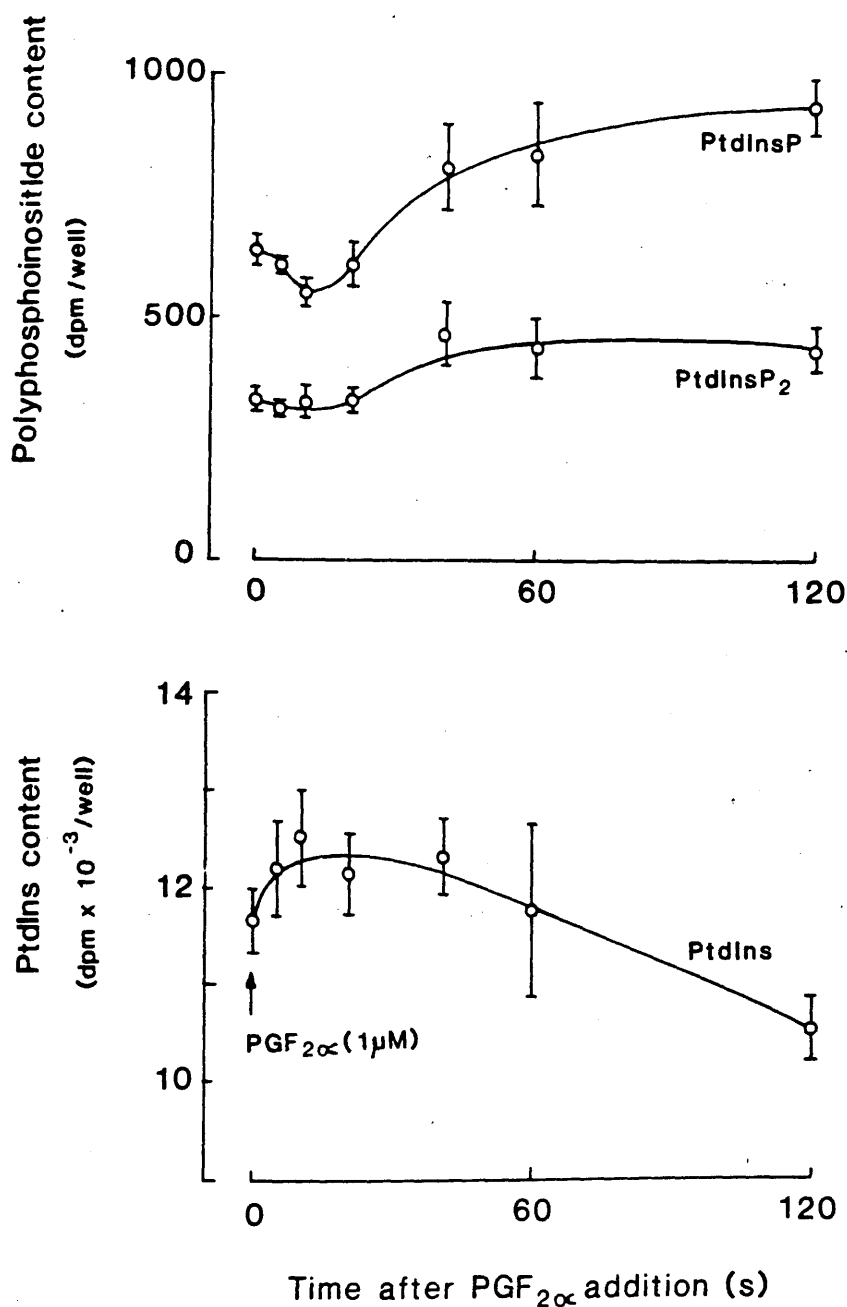


Figure 9: The early time course of the effect of PGF₂α on the inositol phospholipids

Cells were labelled to equilibrium with 5 μCi [³H]-inositol/well. 10 mM LiCl-containing BSS throughout the experiment and samples were incubated with 1 μM PGF₂α. For other experimental details see Methods section. The values shown are from a representative experiment carried out in quadruplicate and replicated once.

1.6 μM $\text{PGF}_{2\alpha}$ did not significantly affect the level inositol over the 20 minutes of the incubation (data not shown). In contrast, the levels of InsP almost doubled within 40s of administration of $\text{PGF}_{2\alpha}$, and fell toward, but did not reach, basal within the 20 minutes stimulation with $\text{PGF}_{2\alpha}$ (Figure 10). Likewise, InsP_2 and InsP_3 levels rose within 40s of administration of $\text{PGF}_{2\alpha}$ and fell, reaching basal levels, with the 20 minute period monitored (Figures 11 and 12). The radioactivity measured in the InsP_4 fraction was too low to record any significant changes associated with the administration of $\text{PGF}_{2\alpha}$ (data not shown).

In an experiment where earlier time points were investigated the same overall pattern emerged. $\text{PGF}_{2\alpha}$ (1 μM) again, did not affect the level of cellular inositol (data not shown). The level of InsP (Figure 13a), as before, increased to a maximum within 40-60s after administration of $\text{PGF}_{2\alpha}$. This increase was maintained over the 2 min duration of the drug incubation. InsP_2 levels (Figure 13b) increased to a maximum after 20s with $\text{PGF}_{2\alpha}$ and fell back towards basal within the 2 min. InsP_3 levels (Figure 13c) rose very rapidly to reach a maximum within 5s of administration with $\text{PGF}_{2\alpha}$. This rise was maintained for up to 40s, whereafter, the level of InsP_3 fell back towards basal, again within the 2 min stimulation with $\text{PGF}_{2\alpha}$.

3.1.1.5 HPLC analysis of the acid-soluble [^3H]-inositol metabolites in $\text{PGF}_{2\alpha}$ -stimulated Swiss 3T3 cells

High performance liquid chromatographical (HPLC) analysis of the acid-soluble [^3H]-inositol metabolites was conducted

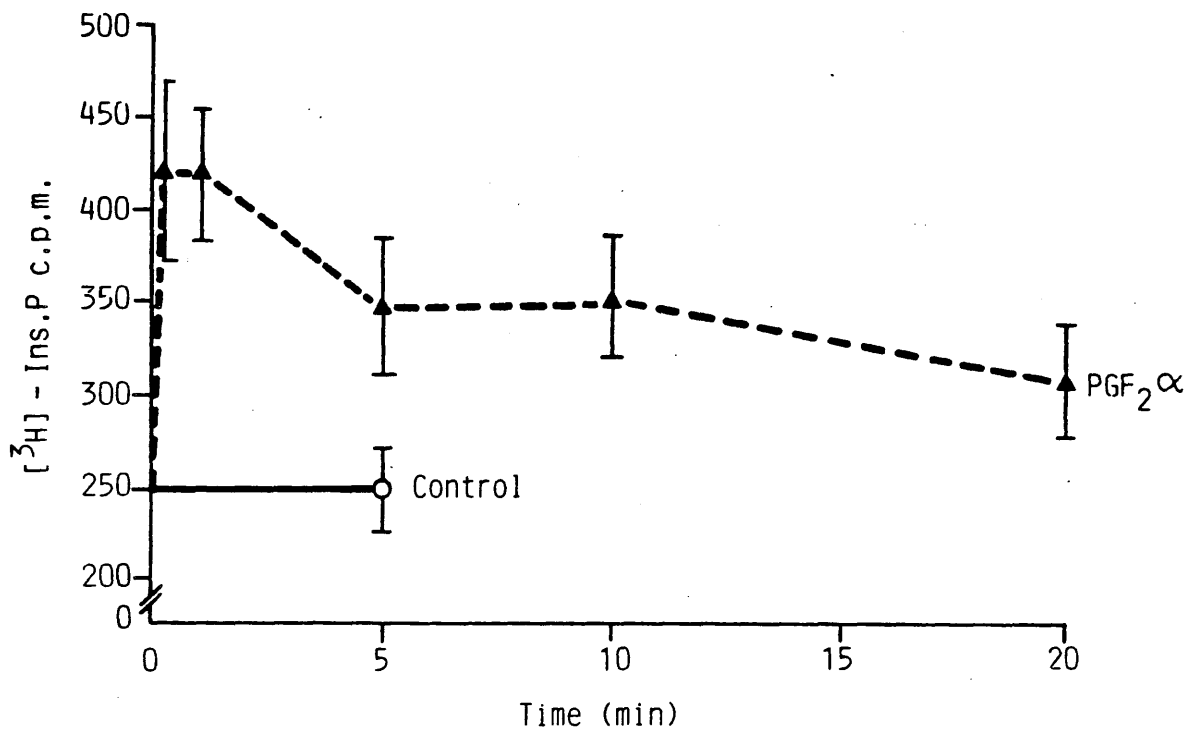


Figure 10: The time course of the effect of PGF₂α on [3H] -
InsP levels in Swiss 3T3 cells

Cells were labelled to equilibrium with 5 μ Ci [3H] -inositol/dish. 10 mM LiCl-containing BSS was used throughout the experiment and samples were incubated with 1.6 μ M PGF₂α. For other experimental details see Methods section. The values shown above are from a representative experiment carried out in duplicate and replicated twice.

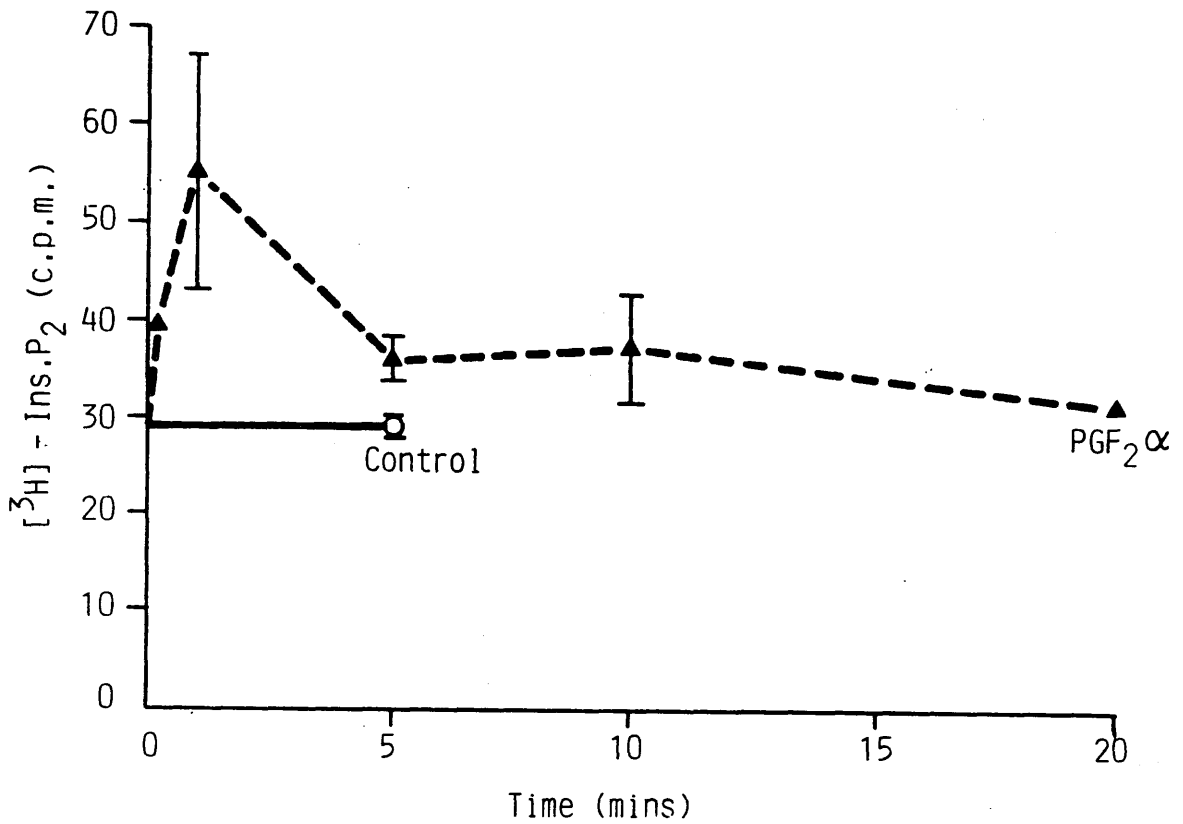


Figure 11: The time course of the effect of PGF₂α on [3H]-
-InsP₂ levels in Swiss 3T3 cells.

Cells were labelled to equilibrium with 5 μ Ci [3H] -inositol/dish. 10 mM LiCl-containing BSS was used throughout the experiment and samples were incubated with 1.6 μ M PGF₂α. For other experimental details see 2α Methods section. The values shown above are from a representative experiment carried out in duplicate and replicated twice.

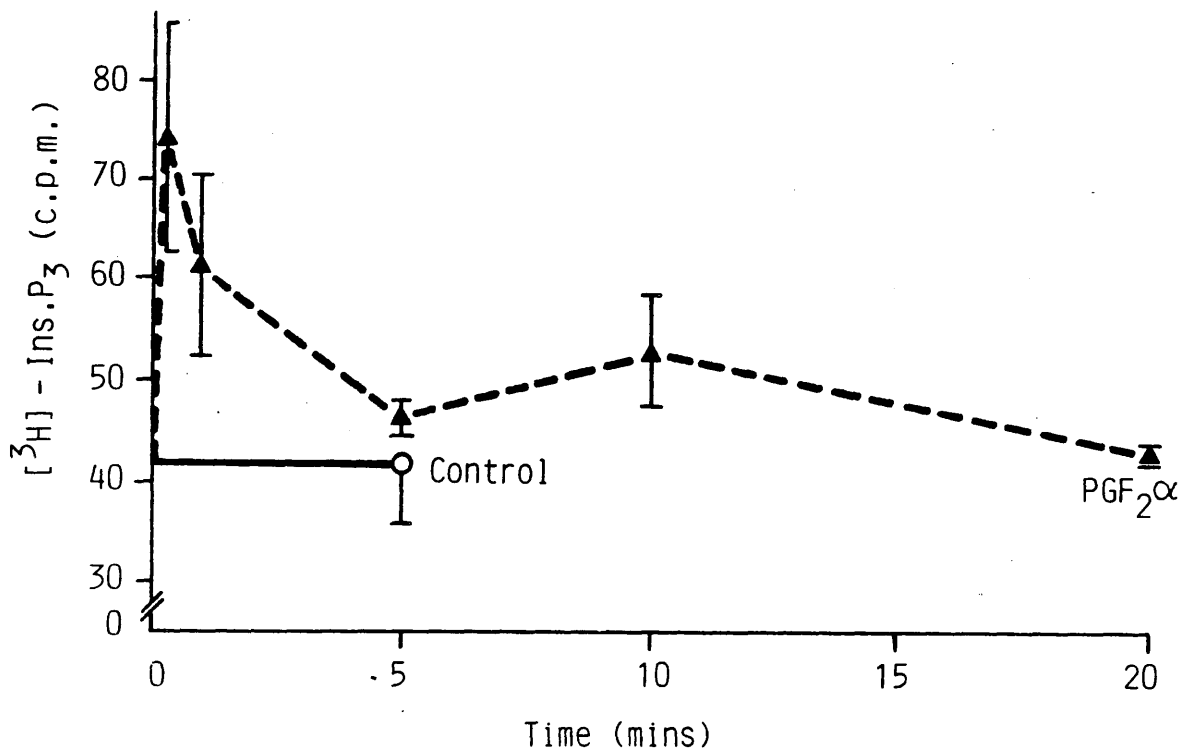


Figure 12: The time course of the effect of PGF₂α on [3H] - InsP₃ levels in Swiss 3T3 cells.

Cells were labelled to equilibrium with 5 μ Ci [3H] -inositol/dish. 10 mM LiCl-containing BSS was used throughout the experiment and samples were incubated with 1.6 μ M PGF₂α. For other experimental details see Methods section. The values shown above are from a representative experiment carried out in duplicate and replicated twice.

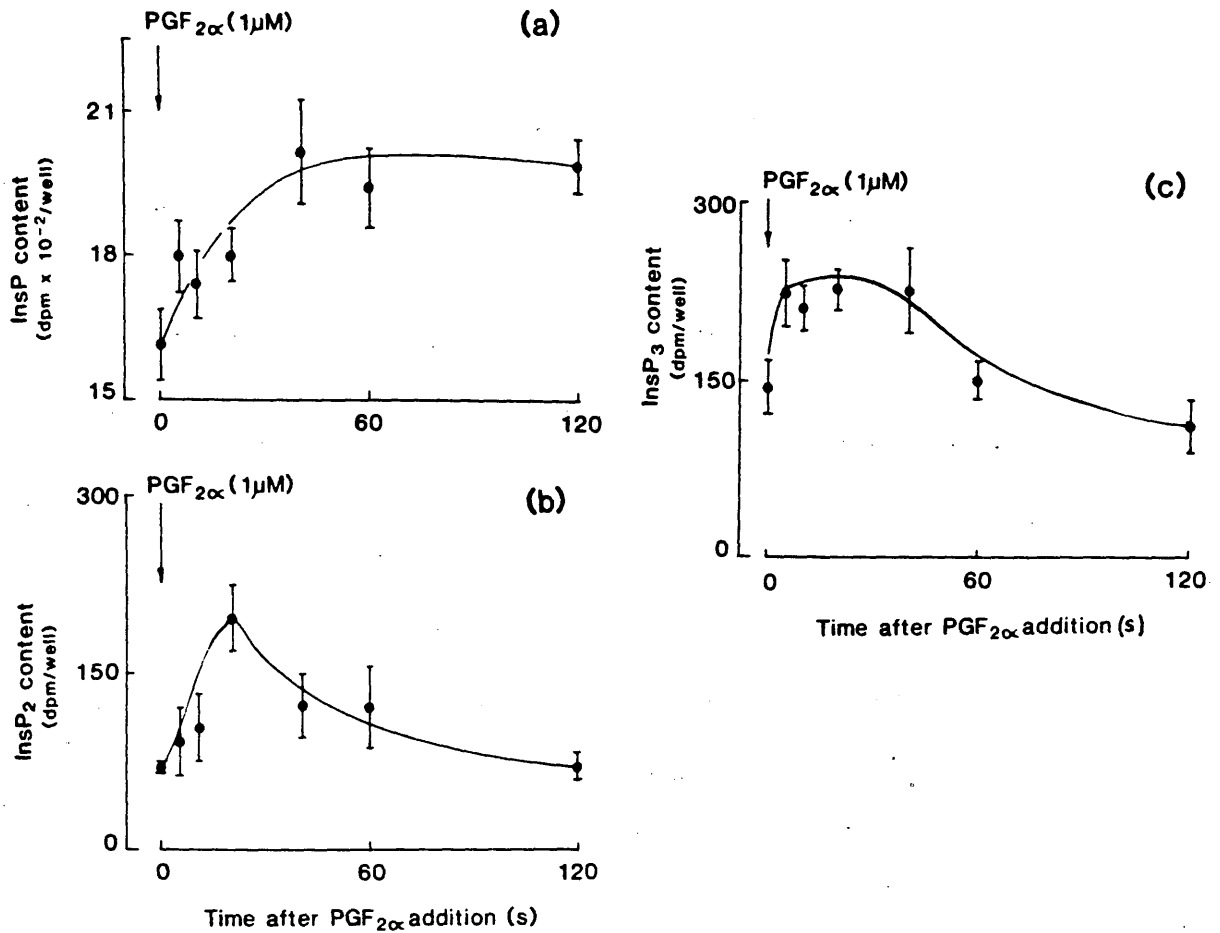


Figure 13: The early time course of the effect of $\text{PGF}_{2\alpha}$ on the inositol phosphates.

Cells were labelled to equilibrium with 5 μCi [^3H]-inositol/well. 10 mM LiCl-containing BSS was used throughout the experiment and samples were incubated with 1 μM $\text{PGF}_{2\alpha}$. For other experimental details see Methods section. The values shown are from a representative experiment carried out in quadruplicate and replicated once.

(a) InsP: (b) InsP₂: (c) InsP₃.

in an attempt to further elucidate the mechanisms underlying stimulated inositol lipid metabolism in Swiss 3T3 cells. This was made difficult due to the extremely high levels of [^3H] -inositol found in Swiss 3T3 cells, and required that the Partisil SAX column be extensively washed with distilled H_2O prior to starting the salt gradient. However, the results shown in Figure 14 indicate clearly, that $\text{Ins}(1,4)\text{P}_2$ levels rise in response to $\text{PGF}_{2\alpha}$ ($1\ \mu\text{M}$). There does not appear to be a significant amount of $\text{Ins}(4,5)\text{P}_2$ present in resting or stimulated 3T3 cells, although, there may be some $\text{Ins}(3,4)\text{P}_2$, seen in Figure 14 as the "shoulder" between the two InsP_2 standards. The shoulder elutes close to $\text{Ins}(3,4)\text{P}_2$ which has been chemically identified as the second-eluting InsP_2 in GH_3 cell extracts (Drummond, A.H., personal commun.). In resting Swiss 3T3 cells, the $\text{Ins}(1)\text{P}$ isomer is the most abundant monophosphate, but it appears that some $\text{Ins}(4)\text{P}$ may also be present. In $\text{PGF}_{2\alpha}$ -stimulated cells the $\text{Ins}(1)\text{P}$ content is significantly increased, but not the $\text{Ins}(4)$ content.

Unfortunately, these samples were run on an ageing HPLC column and the recovery of [^3H] - $\text{Ins}(1,4,5)\text{P}_3$ from this column was only about 20%. Because of this it was impossible to detect the nature of the [^3H] - InsP_3 isomers present in the Dowex elutions. Also, only the region of the gradient corresponding to 0 to about 0.9M ammonium formate is shown in Figure 14. No further counts were detected in this series of experiments that might correspond, for example, to InsP_4 or higher polyphosphates (data not shown).

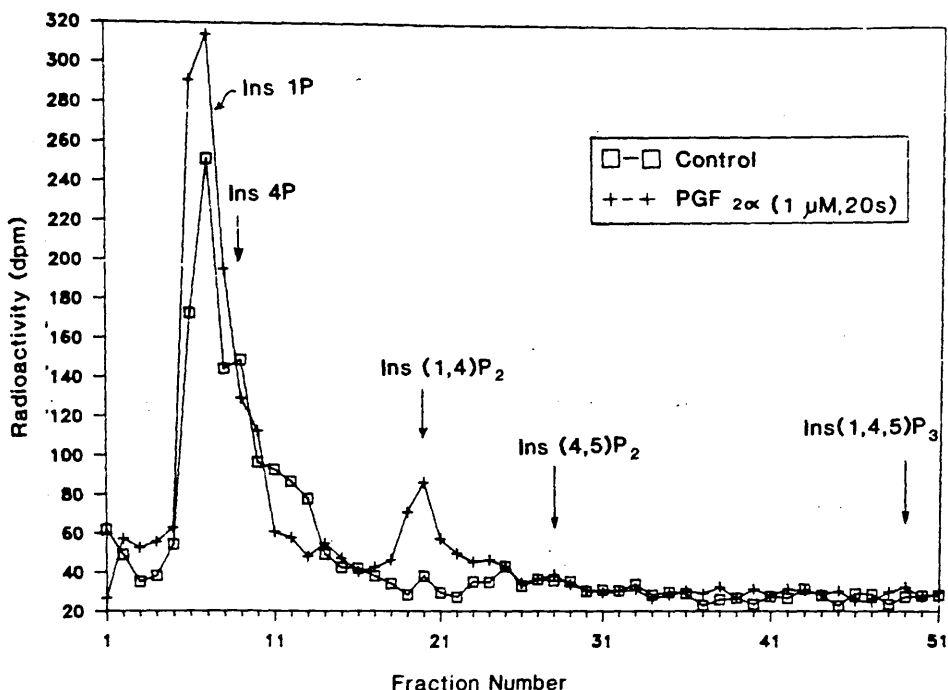


Figure 14: HPLC analysis of the inositol phosphates in the presence and absence of PGF₂α in Swiss 3T3 cells

Cells were labelled to equilibrium with 5 μ Ci [3 H] -inositol. 10 mM LiCl-containing BSS was used throughout the experiment and samples were incubated for 20s with 1 μ M PGF₂α. For experimental details see Methods₃ section. The above data are from a representative experiment conducted in triplicate and repeated once.

3.1.2 Studies using Swiss 3T3 cells pre-labelled with [^3H] - glycerol

The effect of $\text{PGF}_{2\alpha}$ on the 3T3 cell content of the other major phospholipids was investigated using cells pre-labelled to equilibrium with [^3H]-glycerol. The data in Table 5 show the level of phosphatidic acid (PtdA), phosphatidylinositol (PtdIns), phosphatidylserine (PtdS), phosphatidylethanolamine (PtdE) and phosphatidylcholine (PtdCho) in 3T3 cells in response to stimulation with $\text{PGF}_{2\alpha}$ ($1.6 \mu\text{M}$) for 10 min. From this table, it is evident that there was no significant change in the levels of PtdIns, PtdS, PtdE or PtdCho. However, the cellular content of PtdA more than doubled in response to $\text{PGF}_{2\alpha}$. The relative amounts of the phospholipids in 3T3 cells appear to be (PtdCho : PtdE : PtdS : PtdIns : PtdA) 40 : 33.5 : 13 : 12.5 : 1. This agrees with other cell types (e.g. GH_3 cells, Drummond, A.H., personal commun.) where PtdCho and PtdE are the major phospholipids present. Although not measured, other phospholipids appeared to be present in 3T3 cells. Sphingomyelin, lyso PtdIns and cardiolipid were the most evident of these, as identified by their co-migration with standards.

The response in PtdA to $\text{PGF}_{2\alpha}$ was examined further, by measuring the cellular content of PtdA at various time points, following stimulation with $\text{PGF}_{2\alpha}$. The data in Figure 15 reveal that the level of PtdA, like the polyphosphoinositides and inositol phosphates, increases to a maximum within 40s of administration and is maintained over the 20 min stimulation with $\text{PGF}_{2\alpha}$ ($1.6 \mu\text{M}$).

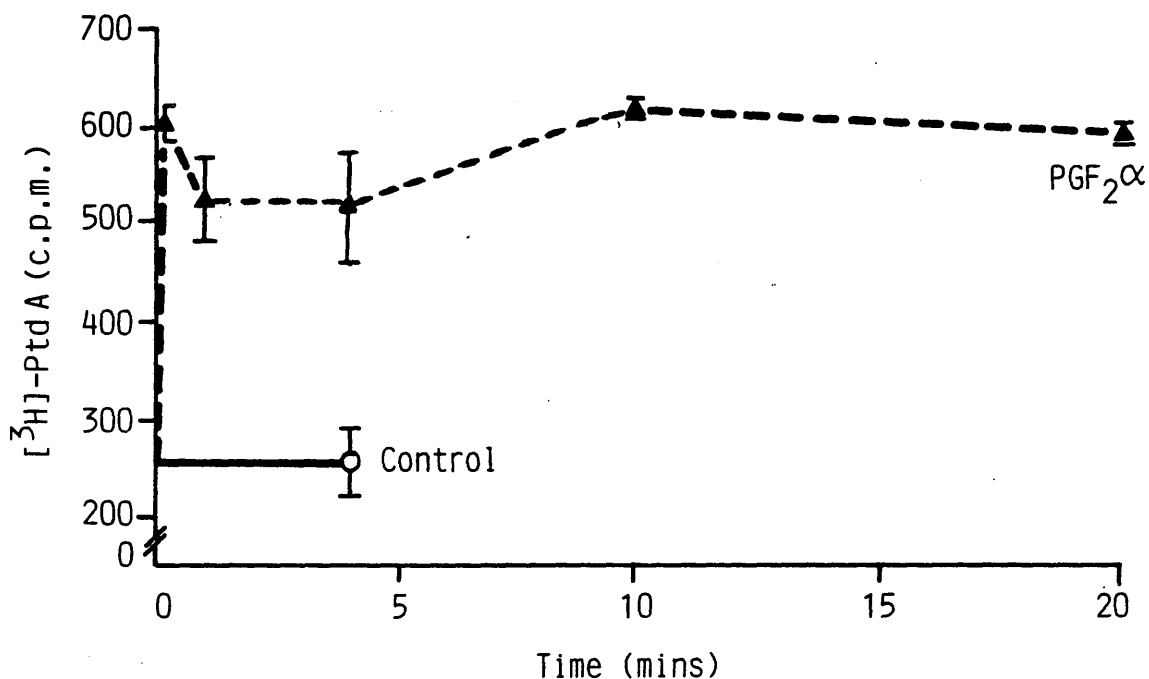


Figure 15: The time course of the effect of PGF₂α on [³H] - PtdA levels in Swiss 3T3 cells

Cells, labelled to equilibrium with 40 μ Ci [³H]-glycerol/dish, were stimulated with 1.6 μ M PGF₂α. For experimental details, see Methods section. The data shown are from a representative experiment conducted in triplicate and replicated once. In this experiment the control samples were taken only at 0 and 5 min. However, from previous experiments it was observed that the PtdA content in control cells did not change significantly over the 20 min. measured (data not shown).

In summary, a supramaximal concentration of $\text{PGF}_{2\alpha}$ leads to rapid stimulation of the inositol lipid cycle in Swiss 3T3 fibroblasts. The response is classical in many aspects, although the rate of stimulation appears to wane rapidly in the first minute after addition of $\text{PGF}_{2\alpha}$. Noteworthy, are the rapid increase in polyphosphoinositide levels that follows agonist addition and the high levels of inositol in 3T3 cells. This precludes the detection of the increased flux through the sugar that must occur in stimulated cells and makes it likely that many days of stimulation in the presence of lithium would be necessary to deplete the cellular content significantly.

In the next section the physiological relevance of the effects of $\text{PGF}_{2\alpha}$ on the major phospholipids and on the inositol lipid cycle is examined.

3.2 The effects of arachidonic acid on inositol lipid metabolism in Swiss 3T3 cells

As previously stated in the introduction, platelet-derived growth factor (PDGF) stimulates both inositol lipid metabolism and the formation of $\text{PGF}_{2\alpha}$, PGE_2 and PGI_2 in Swiss 3T3 cells (Habenicht et al., 1981; Berridge et al., 1984a; Hasegawa-Sasaki, 1985).

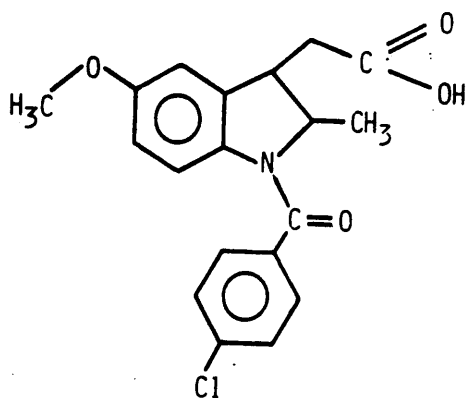
Thus, in view of the fact that $\text{PGF}_{2\alpha}$ and PGE_2 stimulate inositol lipid metabolism (Macphee et al., 1984), it seemed possible that the prostaglandins might be intermediary in the effect of PDGF on inositol lipid metabolism. The results in Section 3.1, clearly show that $\text{PGF}_{2\alpha}$ causes an increase in the turnover of the inositol lipid cycle. Also,

studies in a number of tissues including blood platelets (Seiss et al., 1983) have revealed the involvement of cyclooxygenase products in the action of primary agonists that can stimulate inositol lipid metabolism. A number of possible mechanisms of action of PDGF are shown in Figure 5 and discussed in Section 1.3. In Section 3.3, the effects of serum and PDGF on Swiss 3T3 cells were investigated. But first, the possible involvement of the prostaglandins in the action of PDGF was studied. To do this, it was necessary to be able to block their synthesis by inhibiting the cyclooxygenase enzyme (see Section 1.1.6).

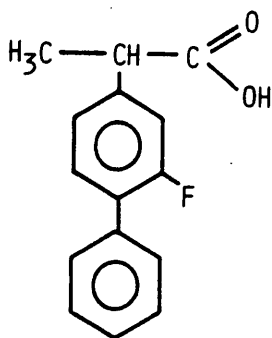
Two cyclooxygenase inhibitors were chosen. Flurbiprofen (Figure 16) is a water soluble compound which is reported to be more potent in inhibiting cyclooxygenase than the other cyclooxygenase inhibitor used, indomethacin (Brodgen et al., 1979 and Figure 16).

3.2.1 The effects of flurbiprofen and indomethacin on inositol lipid metabolism in Swiss 3T3 cells

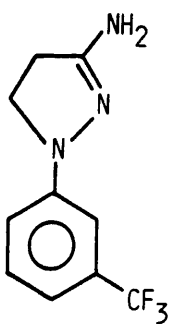
In the first set of experiments, cells were labelled with 40 μCi [^3H] -glycerol/dish and incubated for 5 min with 30 μM AA, 20 μM flurbiprofen or 1.6 μM $\text{PGF}_{2\alpha}$. Some cells were first incubated with flurbiprofen for 5 min followed by 5 min incubation with AA. From the data presented in Figure 17, it is clear that 30 μM AA can elicit a rise in PtdA levels. However, rather surprisingly flurbiprofen alone caused an even greater increase in cellular PtdA levels. In cells given a 5 min pre-incubation with flurbiprofen, before addition of AA, there was an increase in PtdA levels



Indomethacin



Flurbiprofen



BW755C

Figure 16: The chemical structure of indomethacin, flurbiprofen and BW755C.

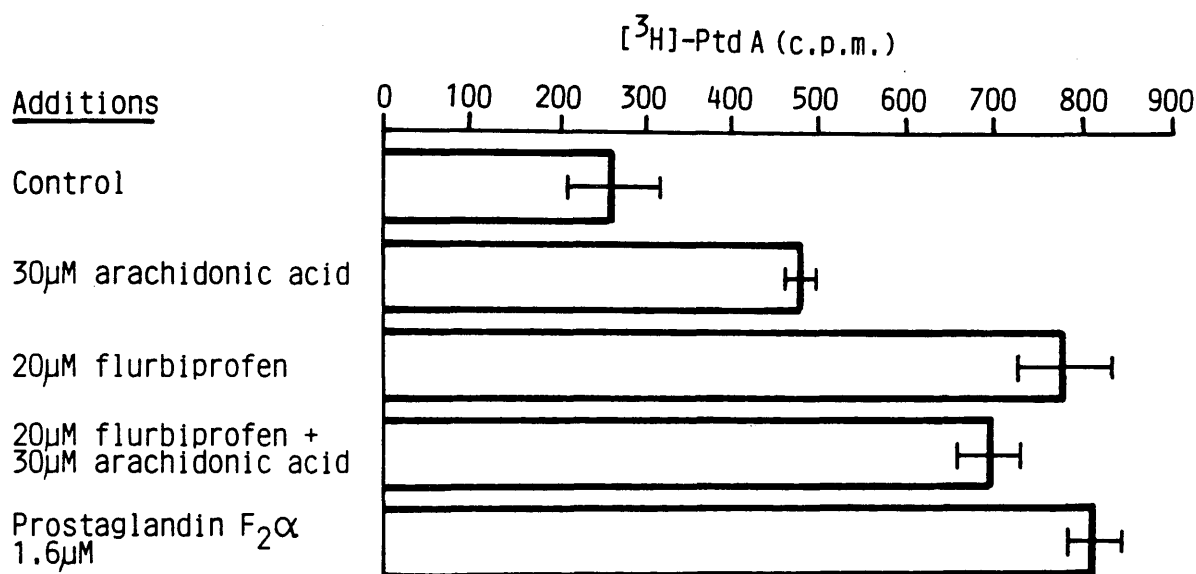


Figure 17: The effect of flurbiprofen on [³H] -PtdA levels in Swiss 3T3 cells.

Cells were labelled to equilibrium with 40 µCi [³H] -glycerol/dish. Drug incubations were for 5 min. In the case of samples receiving both 20 µM flurbiprofen and 30 µM AA, the cells received the flurbiprofen for 5 min. before the AA. The data shown are from a representative experiment conducted in triplicate and repeated twice.

similar increase in PtdA levels to that recorded with flurbiprofen on its own. $\text{PGF}_{2\alpha}$, used as a positive control in this experiment, gave a similar increase in PtdA levels to that recorded with flurbiprofen. There was no significant change in the levels of the other phospholipids measured, (PtdIns, PtdS, PtdCho, PtdE), for any of the above agonists (data not shown).

Obviously, the question arose as to why flurbiprofen itself caused this marked increase in cellular PtdA content and it seemed important to investigate this phenomenon further. It was proposed that the effects of flurbiprofen and indomethacin on inositol lipid metabolism and on AA release from 3T3 cells should be studied.

However, before these experiments could be conducted, it was necessary to know the concentration of flurbiprofen required to give its maximum effect, and whether this was still within the range in which the drug could be envisaged to be acting specifically as a cyclooxygenase inhibitor (up to approximately 300 μM , MacIntyre, D.E., personal commun.).

The data in Figure 18 show the effect of increasing concentrations of flurbiprofen on $[\text{}^3\text{H}]$ -InsP levels. It can be seen from this graph that an increase in InsP resulted from the addition of 3 μM flurbiprofen and that InsP levels rose in a dose-related manner to reach a maximum between 10 and 100 μM flurbiprofen.

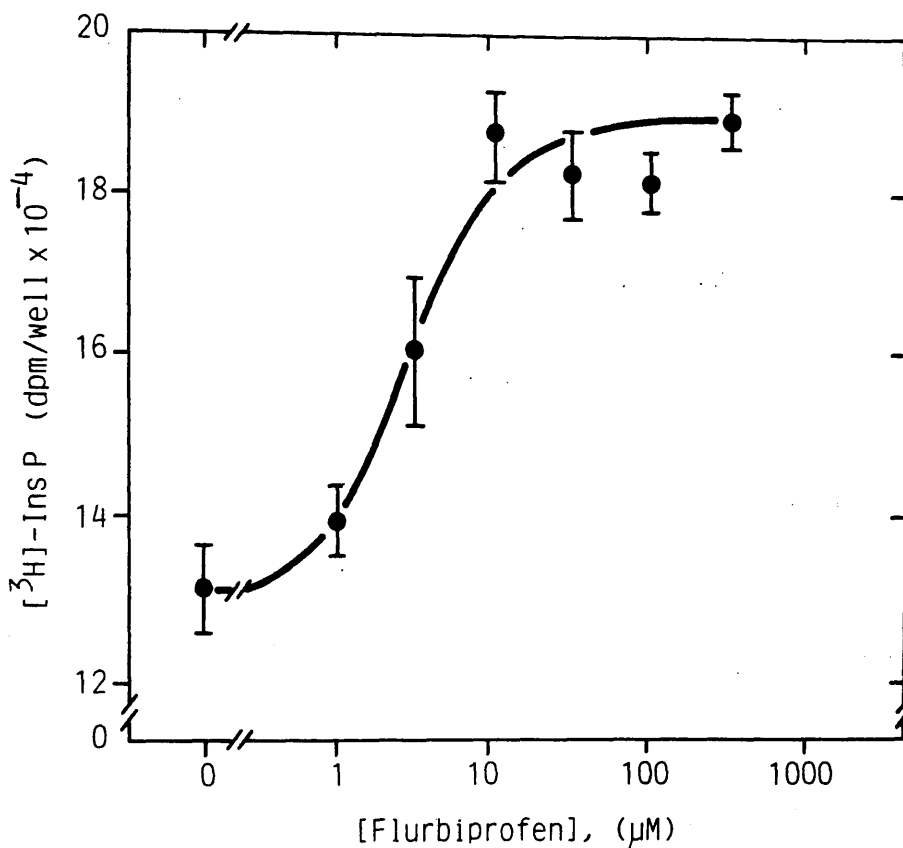


Figure 18: The effect of increasing concentrations of flurbiprofen on [³H] -InsP levels in Swiss 3T3 cells.

Cells were labelled to equilibrium with 5 μCi [³H] -inositol/well. Samples were incubated for 10 min. with flurbiprofen. For experimental details see Methods section. The data shown is from a representative experiment conducted in triplicate and replicated once.

3.2.1.1 The effects of cyclooxygenase inhibitors on the release of [^3H] -AA-derived radioactivity and on [^3H] -arachidonyl PtdA formation in Swiss 3T3 cells

It was expected that flurbiprofen would prevent the metabolism of AA to more polar products. Although in the event that 3T3 cells contained significant levels of lipoxigenase enzymes it was considered possible that AA might be redirected towards this pathway (Figure 4, and Hamberg, 1976). Thus, in cells labelled to equilibrium with 0.5 μCi [^3H] -AA/dish or well, measurements of the release of [^3H] -AA-derived radioactivity into the bathing medium and [^3H] -arachidonyl PtdA formation in cells were carried out.

Concentrations of flurbiprofen which stimulate the inositol lipid cycle in Swiss 3T3 cells also enhance the accumulation of radioactivity in the medium of [^3H] -AA-labelled cells. Table 6 shows that treatment with 100 μM flurbiprofen led almost to a doubling in the accumulation of [^3H] -AA in the medium and this increase was accompanied by the formation of [^3H] -arachidonyl PtdA. Similar results were found with indomethacin, and these are shown in Table 7.

When the time course of these effects was investigated (Figure 19), it was clear that with 30 μM flurbiprofen, [^3H] -AA-derived radioactivity in the medium increased within 10 min and continued increasing, albeit at a markedly reduced rate, between 10 and 40 min. The accumulation of [^3H] -arachidonyl PtdA in response to 30 μM flurbiprofen (Figure 20) peaked at 10 min and then fell back reaching basal levels within 25-40 min.

Agent	[³ H]-arachidonate in the medium (cpm/well)	[³ H]-arachidonyl PtdA (cpm/well)
Control	405 ± 32	4412 ± 375
Flurbiprofen (10μM)	604 ± 27	5984 ± 242
Flurbiprofen (100μM)	788 ± 48	7931 ± 250
PGF _{2α} (1 μM)	625 ± 49	8869 ± 328

(± S.E.M., n = 5)

Table 6: The effect of flurbiprofen on [³H] -AA release and on [³H] -arachidonyl PtdA levels in Swiss 3T3 cells

Cells were labelled to equilibrium with 0.5 μCi [³H] -AA/well. Drug incubations were for 10 min. For experimental details see Methods section. The above data is from 2 experiments, one conducted in duplicate, one in triplicate.

Agent	[³ H] -AA-derived radioactivity in the medium (c.p.m./ well)	[³ H] -arachidonyl PtdA (c.p.m./ well)
EtOH (control)	819 ± 21	2280 ± 37
Indomethacin (0.1 µM)	1073 ± 63	2634 ± 159
Indomethacin (1 µM)	1146 ± 78	3411 ± 140

Table 7: The effect of indomethacin on [³H] -AA-derived radioactivity released into the medium and on [³H] -arachidonyl PtdA formation in Swiss 3T3 cells

Cells were labelled to equilibrium with 0.5 µCi/well [³H] -AA. Samples were incubated for 10 min with indomethacin. For experimental details see Methods Section.

The above data are from two experiments, both conducted in triplicate.

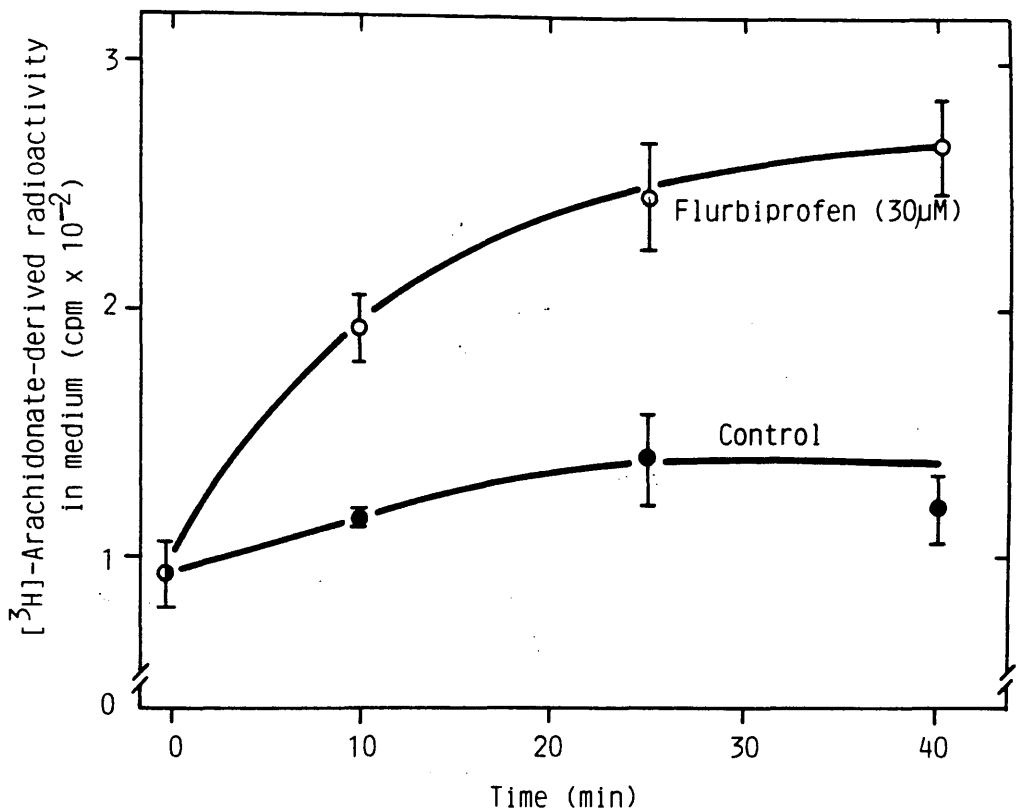


Figure 19: The time course of the effect of flurbiprofen on $[^3\text{H}]$ -arachidonate-derived radioactivity in the medium surrounding Swiss 3T3 cells

Cells were labelled to equilibrium with 0.5 μCi $[^3\text{H}]$ -AA/well, and incubated with 30 μM flurbiprofen. The volume of medium per sample was 2 ml. The above data are from a representative experiment conducted in triplicate and repeated once.

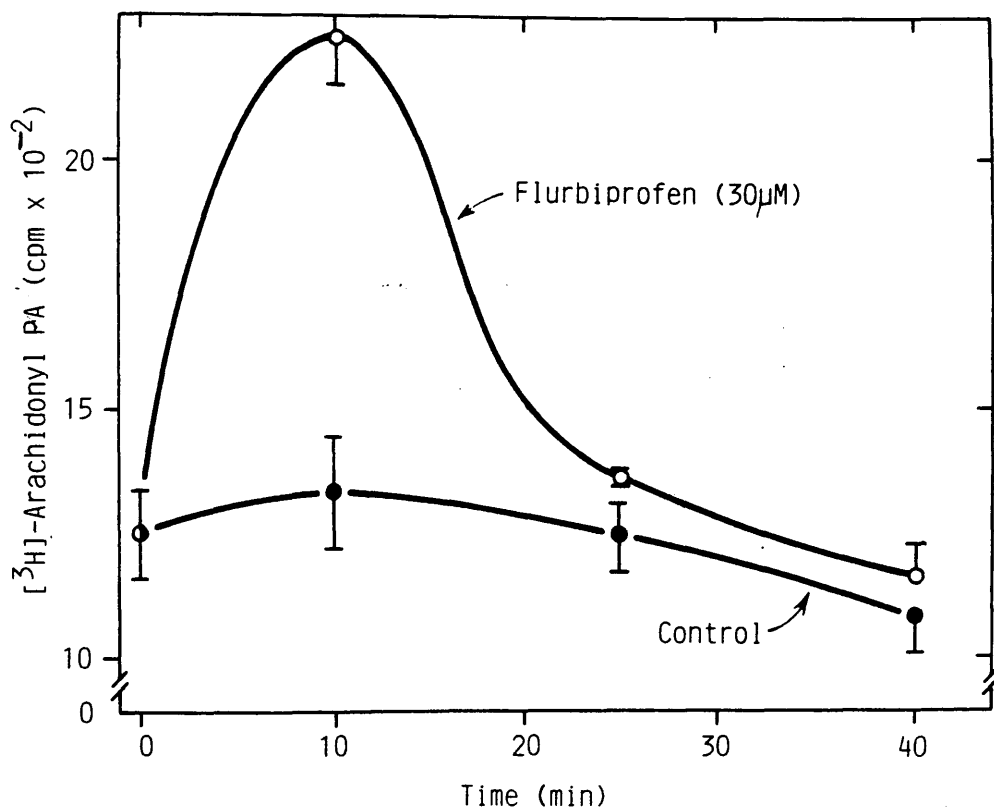


Figure 20: The time course of the effect of flurbiprofen on [³H] -arachidonyl PtdA levels in Swiss 3T3 cells.

Cells were labelled to equilibrium with 0.5 μCi [³H] -AA/well, and incubated with 30 μM flurbiprofen. The volume of medium per sample was 2 ml. The above data are from a representative experiment conducted in triplicate and repeated once.

It is evident from these data that the formation of [^3H] - arachidonyl PtdA and, to a lesser extent, the release of [^3H] - AA, stimulated by flurbiprofen, is relatively short lived. The reason for this is unclear, although it is possible that since phospholipase A_2 can be inhibited by unsaturated fatty acids (Ballou and Cheung, 1985), a negative feedback loop may be operating.

3.2.2 The effects of BW755C, a lipoxygenase inhibitor, on inositol lipid metabolism in Swiss 3T3 cells

The relatively low concentrations of both flurbiprofen and indomethacin that elicit the changes in AA metabolism and inositol lipid metabolism, make it likely that these represent specific drug effects on the cellular cyclooxygenase. It seemed possible, however, that the stimulatory effects of flurbiprofen and indomethacin on inositol lipid metabolism might be due to a redirection of endogenous arachidonic acid (AA) away from cyclooxygenase products and into the lipoxygenase pathway. This occurs in a number of tissues such as smooth muscle, when a cyclooxygenase inhibitor is applied (Hamberg, 1976). In this event a leukotriene or other lipoxygenase product might be the agent which ultimately stimulates the inositol lipid cycle in 3T3 cells.

To investigate this possibility, the effects of the water-soluble compound BW 755C (3-amino-1- m-(trifluoromethyl) phenyl -2-pyrazoline) were investigated. This drug blocks both cyclooxygenase and the 5-, 12- and 15-lipoxygenase enzymes (Needleman et al., 1986) (Figure 16).

Table 8 shows the effects of 1, 10 and 100 μ M BW755C on InsP levels. Although not shown here, the concentration which gave the maximum response was about 100 μ M (in other experiments 300 μ M BW755C did not cause a significantly greater increase in InsP levels than 100 μ M).

3.2.2.1 The effects of BW755C on the release of [3 H] -AA-derived radioactivity and on [3 H] -arachidonyl PtdA formation in Swiss 3T3 cells

From the data shown in Figure 21, it is clear that the concentration of BW755C which gave the maximum increase in InsP levels (100 μ M), also gave the maximum increase in the release of [3 H] -AA-derived radioactivity into the bathing medium. This dose-response relationship is paralleled in Figure 22 which shows the effect of increasing concentrations of BW755C on [3 H] -arachidonyl PtdA formation.

Thus, the results of these experiments render it unlikely that a stimulatory lipxygenase metabolite is involved in the effect on inositol lipid metabolism mediated by the cyclooxygenase blockers. Also, there are no published results indicating that lipxygenase activity has been detected in Swiss 3T3 cells.

Further support for this derives from experiments in which the radioactivity release into the bathing medium was analysed by thin-layer chromatography (see Methods). More than 95% of the radioactivity co-migrated with standard AA whether flurbiprofen was present or not (Figure 23). It is noteworthy from this figure that resting 3T3 cells appear to contain very little prostaglandins.

BW755C μ M	$[^3\text{H}]$ -InsP c.p.m./well
0	174 \pm 15
1	202 \pm 8
10	236.2 \pm 12
100	279.3 \pm 17

Table 8: The effect of BW755C on $[^3\text{H}]$ -InsP formation in Swiss 3T3 cells

Cells were labelled to equilibrium with 5 μ Ci/well $[^3\text{H}]$ -inositol. Samples were incubated for 10 min with BW 755C. The data shown are from two experiments, each conducted in triplicate. The maximum increase in $[^3\text{H}]$ -InsP was observed with 100 μ M BW755C. 300 μ M BW755C did not significantly increase the level of $[^3\text{H}]$ -InsP above that recorded with 100 μ M BW755C (data not shown).

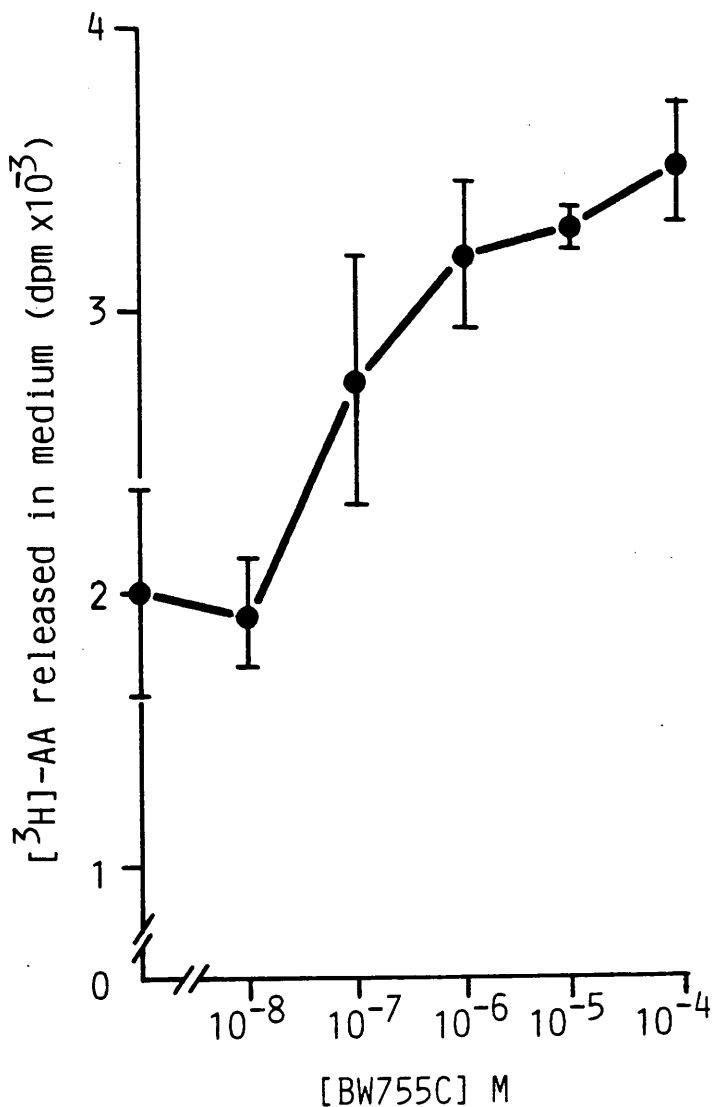


Figure 21: The effect of increasing concentrations of BW755C on [³H] -AA release from Swiss 3T3 cells.

Cells were labelled to equilibrium with 0.5 μ Ci [³H] -AA/well. For experimental details, see Methods section. Samples were incubated for 10 min. with BW755C. The data shown are from a representative experiment conducted in triplicate and repeated once.

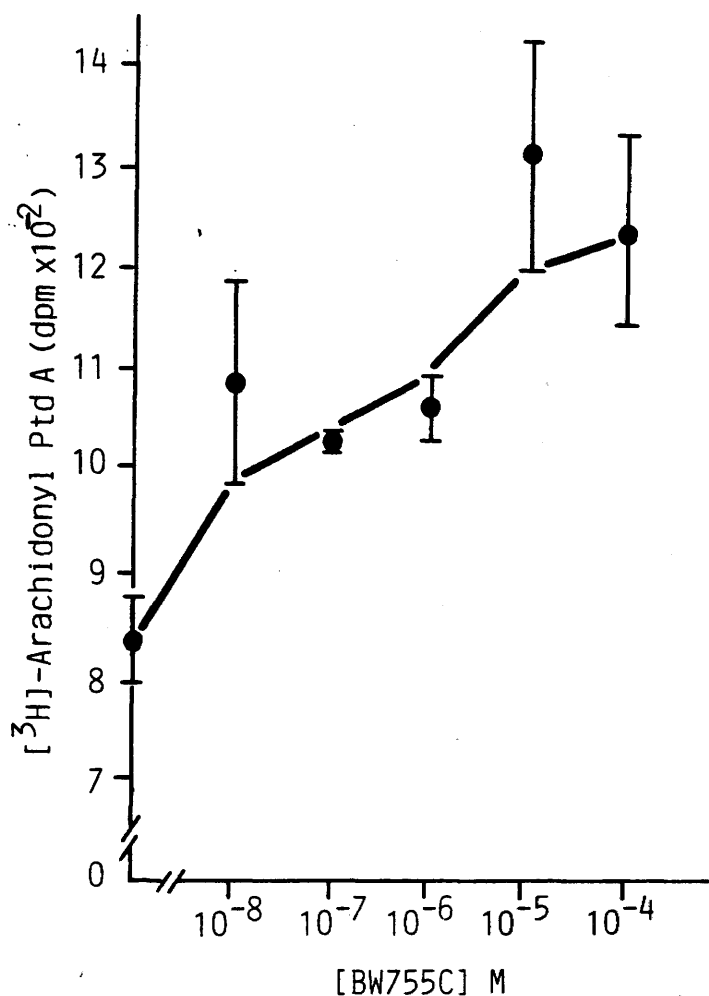


Figure 22: The effect of increasing concentrations of BW755C on [³H] -arachidonyl PtdA levels in Swiss 3T3 cells.

Cells were labelled to equilibrium with 0.5 μ Ci [³H] -AA/well. For experimental details, see Methods section. Samples were incubated for 10 min. with BW755C. The data shown are from a representative experiment conducted in triplicate and repeated once.

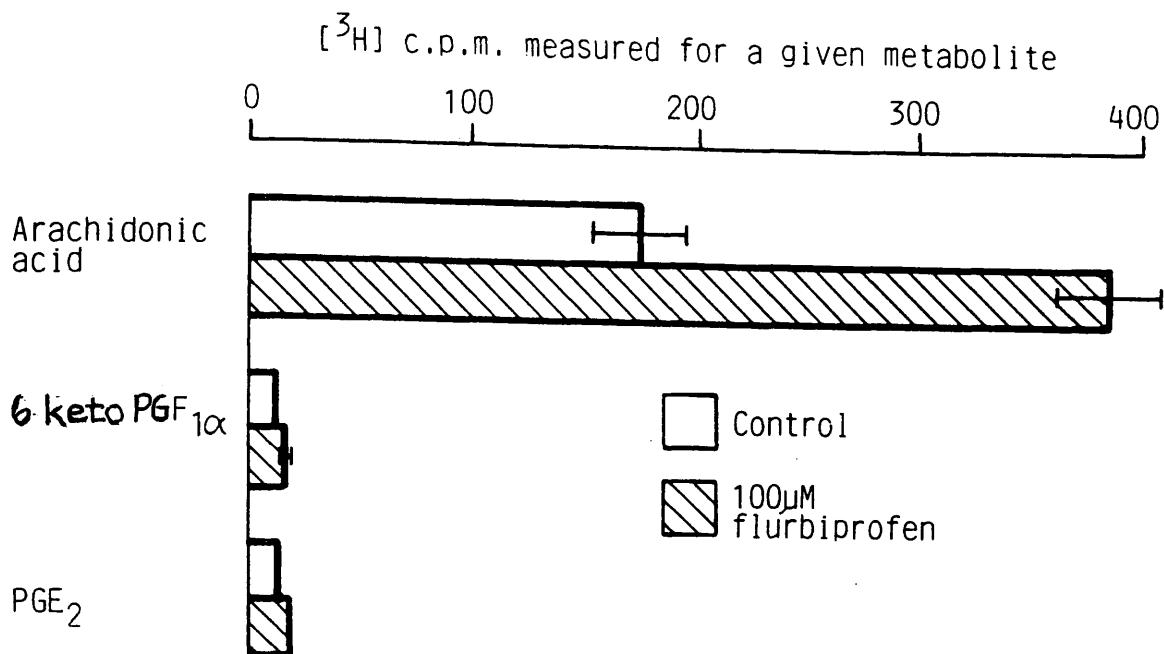


Figure 23: The effect of flurbiprofen on AA, 6 keto PGF_{1α} and PGE₂ content in Swiss 3T3 cells

Cells were labelled to equilibrium with 0.5 μCi [³H] -AA/dish (6 ml). Samples were incubated for 10 min with 100 μM flurbiprofen. For experimental details see Methods section. The data shown are from a representative experiment carried out in triplicate and repeated once.

However, on a cautionary note, it may be that BW755C is not specific enough for all the lipoxygenases and that an "Uncharacterised" stimulatory lipoxygenase metabolite is still formed in the presence of BW755C. This might, in addition, escape detection in the TLC analysis: some hydroxy-acids, for example, co-migrate with AA in this separation (MacIntyre, D.E., unpublished results).

3.2.3 The effects of arachidonic acid on inositol lipid metabolism in Swiss 3T3 cells

The results from the experiments using BW755C and, indeed, from the flurbiprofen studies suggested that changes in the content of arachidonic acid (AA), itself, might be the possible cause of the increase in inositol lipid metabolism. In Figure 24, the graph shows the effect of increasing concentrations of AA on InsP levels, 100 μ M AA gave the maximum effect of about twice the control levels of InsP, although the graph does not show an extension of the dose-response curve (in other experiments 300 μ M AA, shown in Figure 25, gave a similar increase in InsP as 100 μ M AA).

3.2.3.1 The effects of analogues of arachidonic acid on inositol lipid metabolism in Swiss 3T3 cells

In an attempt to show if any structure activity relationships for AA existed, analogues of AA were compared with AA itself. In particular, it was important to determine whether fatty acids which are not subject to metabolism to more polar metabolites e.g. prostaglandins and leukotrienes, were also active in stimulating inositol lipid metabolism in 3T3 cells.

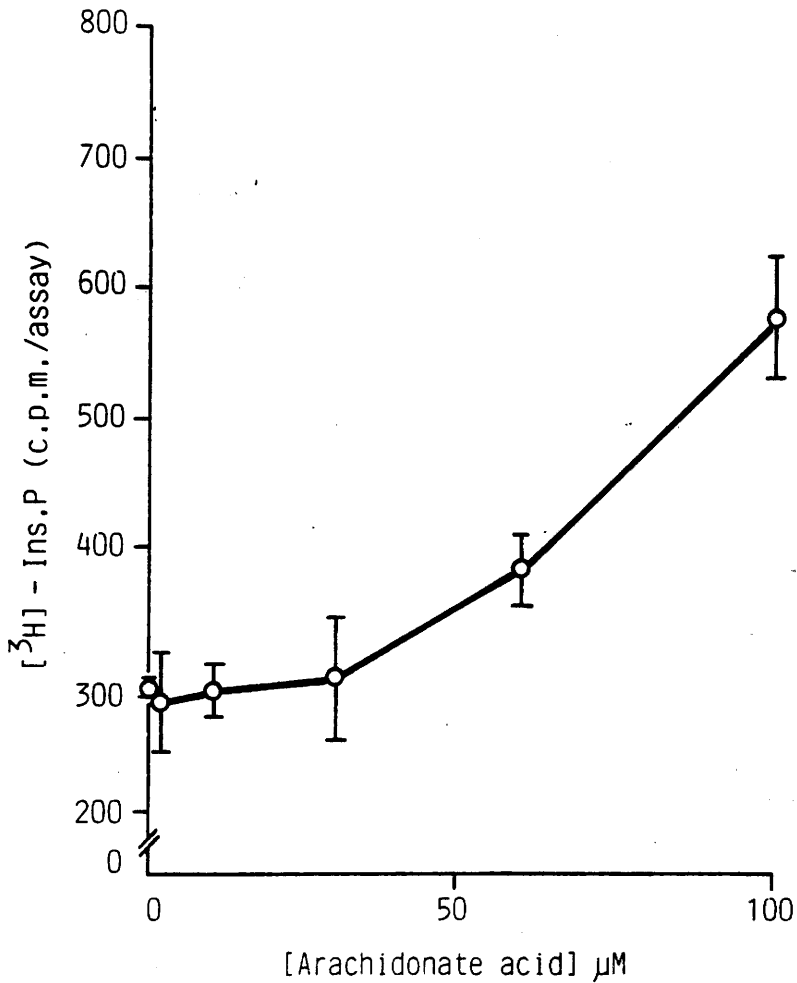


Figure 24: The effect of increasing concentrations of AA on $[\text{}^3\text{H}] - \text{InsP}$ levels in Swiss 3T3 cells

Cells were labelled to equilibrium with 5 μCi $[\text{}^3\text{H}] - \text{inositol/dish}$ (6 ml). Li^+ -containing BSS was used, as in previous experiments. No cyclooxygenase or lipoxygenase blockers were present in this, a representative experiment conducted in triplicate and repeated once.

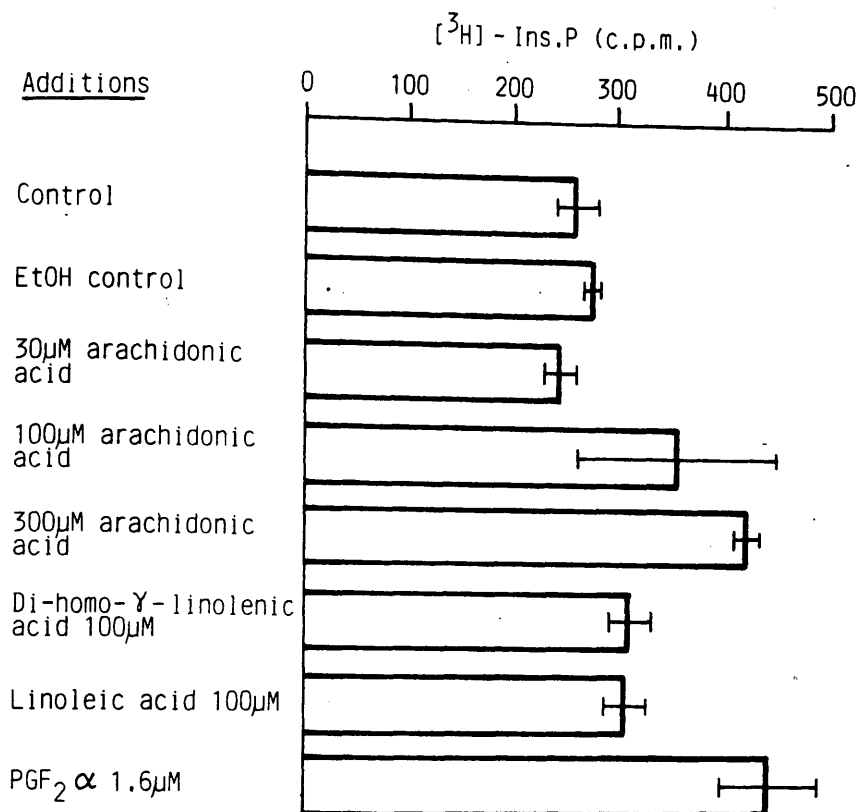


Figure 25: The effect of AA and its analogues on [³H] - InsP levels in Swiss 3T3 cells.

Cells were labelled to equilibrium with 5 µCi [³H]-inositol/dish (6 ml). No cyclooxygenase or lipoxygenase blockers were present in these experiments. Drug incubations were for 10 min. For experimental details see Methods section. The data shown are from an experiment conducted in triplicate and repeated once.

Di-homo- γ -linolenic acid, from which the prostaglandin 1 series is derived and linoleic acid, which is the precursor of AA were the only analogues, tested, to have activity (Figure 25).

Eicosapentaenoic acid, from which the prostaglandin 3 series is derived and eicosatrienoic acid, which is a man made (unnatural) fatty acid had no activity. Not surprisingly, the two analogues of AA which were active are the closest in structure to AA itself. However, as we can see from Figure 25 they are not as potent as AA itself.

3.2.4 The effects of drugs which alter PLA₂ activity in Swiss 3T3 cells

Phospholipase A₂ (PLA₂) is the collective name given to a group of enzymes which hydrolyse membrane phospholipids to yield arachidonic acid (see Section 1.1.6). If the level of intra- or extra-cellular free AA is an important factor in the control of inositol lipid metabolism in 3T3 cells, other agents, which regulate the availability of the fatty acid should be able to influence PtdA formation. Indeed, the best explanation of the data presented so far in this section is that the recruitment of basal PLA₂ or DG lipase (see Section 1.1.6) activity by cyclooxygenase inhibitors leads to the accumulation of AA.

So, with this in mind, studies using drugs which inhibit or stimulate PLA₂ were carried out and the effects of these drugs on inositol lipid metabolism was monitored.

3.2.4.1 The effect of melittin on the release of [^3H] - AA-derived radioactivity and [^3H] -arachidonyl PtdA formation in Swiss 3T3 cells

Melittin is a PLA_2 activator, derived from wasp venom (Argiolas and Pisano, 1983). It has been shown to increase, several fold, the activity of the $\text{Na}^+ - \text{K}^+$ pump in quiescent Swiss 3T3 cells. Like other growth factors, melittin increases the activity of the pump by increasing Na^+ entry into the cell. In contrast, other early responses are not elicited by the toxin. At concentrations that promote ion fluxes, melittin stimulates DNA synthesis in quiescent mouse cells acting synergistically with insulin, EGF and with FDGF. In contrast, melittin does not interact synergistically with either phorbol esters or vasopressin (Rozengurt et al., 1981c).

The data in Table 9 show that a 10 min incubation with 0.2 $\mu\text{g/ml}$ and 1.0 $\mu\text{g/ml}$ melittin stimulates both AA release and arachidonyl PtdA formation in 3T3 cells. These concentrations of melittin do not lead to cell lysis, as judged by the ability of melittin-treated cells to exclude trypan blue (data not shown).

3.2.4.2 The effect of dexamethasone on flurbiprofen- and BW 755C-stimulated Swiss 3T3 cells

The anti-inflammatory glucocorticoid, dexamethasone, was used in some studies to inhibit PLA_2 indirectly. Dexamethasone is thought to act by increasing the synthesis of lipocortin, a protein inhibitor of PLA_2 (see Section 1.1.6).

Agent	[³ H]-arachidonate in the medium (cpm/well)	[³ H]-arachidonyl PtdA (cpm/well)
Control	414 ± 11	1380 ± 187
Melittin (0.2µg/ml)	640 ± 49	4572 ± 643
Melittin (1.0µg/ml)	1307 ± 195	6480 ± 577
(10 min incubation: means ± S.E.M., n= 4)		

Table 9: The effect of melittin on [³H] -AA release and on [³H] -arachidonyl PtdA levels in Swiss 3T3 cells

Cells were labelled to equilibrium with 0.5 µCi [³H] -AA/well (2 ml). Samples were incubated with melittin for 10 min. For experimental details see Methods section. The data is from 2 experiments, each conducted in duplicate.

Firstly, the effect of dexamethasone on flurbiprofen-stimulated 3T3 cells was investigated. The cells were pre-treated with 30 nM dexamethasone for 24 hours prior to the experiment (Figure 26). The results clearly show that dexamethasone abolishes the increase in InsP formation observed in the presence of flurbiprofen.

It must be pointed out here, that the results obtained in experiments with dexamethasone were variable, although not in respect of the dexamethasone itself. In 2 out of 5 experiments no significant difference in InsP levels from controls was noted in response to flurbiprofen, and therefore dexamethasone had no response to abolish. The variability of 3T3 cells to respond to flurbiprofen (and to BW755C) is discussed in Section 4. However, in the majority of experiments the effect of dexamethasone could be seen clearly.

A further example of this is illustrated in Figure 27, where the effect of cell pre-treatment with various concentrations of dexamethasone on BW755C-stimulated cells can be seen. As in the previous series of experiments, the 3T3 cells were pre-treated with dexamethasone for 24 hours. The cells were then stimulated with a saturating concentration of BW755C (100 μ M). The graph shows that a concentration of dexamethasone as low as 0.1 nM significantly reduced (but did not abolish) the increase in InsP observed in the presence of BW755C. Concentrations of dexamethasone of 10 nM and above, clearly abolished the increase in cellular InsP observed in the presence of BW755C.

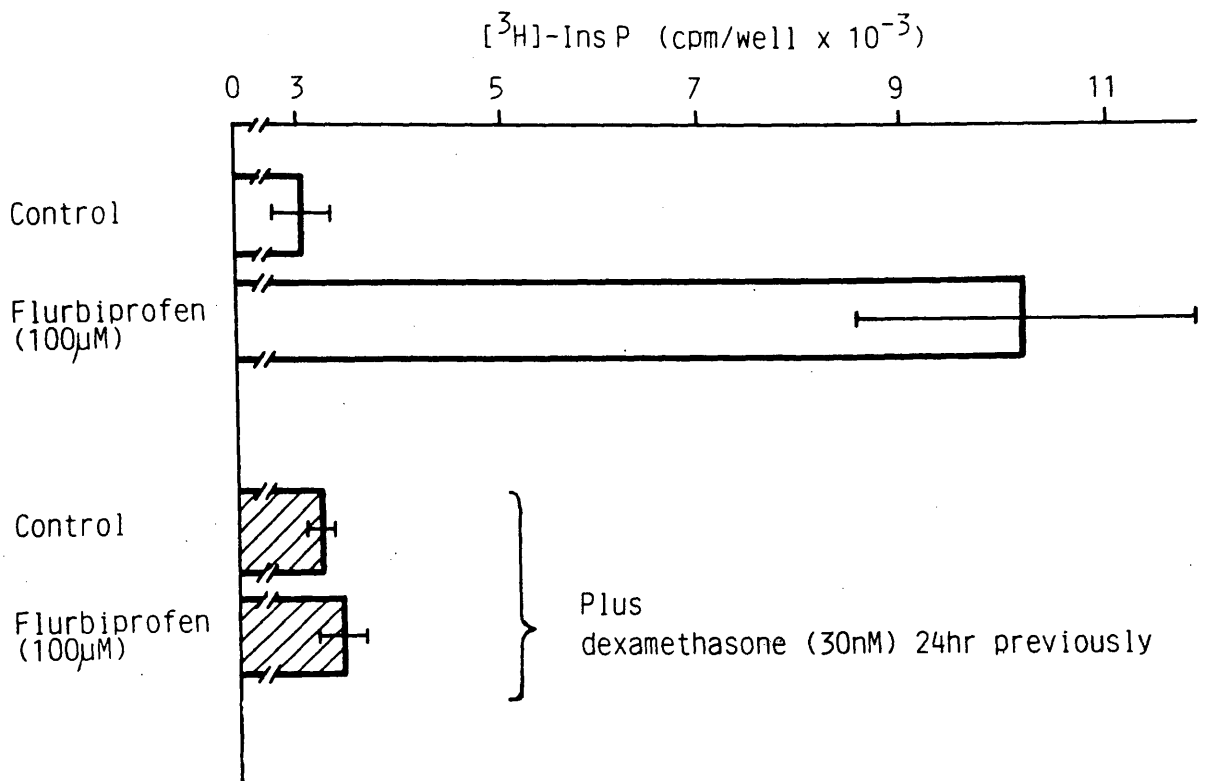


Figure 26: The effect of dexamethasone on the flurbiprofen-induced rise in [³H]-InsP levels in Swiss 3T3 cells.

Cells were labelled to equilibrium with 5 µCi [³H]-inositol/well. Half the samples received 30 nM dexamethasone 24 hours prior to the experiment. The control samples received an equal volume of ethanol. Samples were incubated with flurbiprofen (or 0.9% (w/v) saline) for 10 min. The data shown are from a representative experiment carried out in triplicate and replicated three times (see Text). In two other experiments flurbiprofen had no effect on InsP levels.

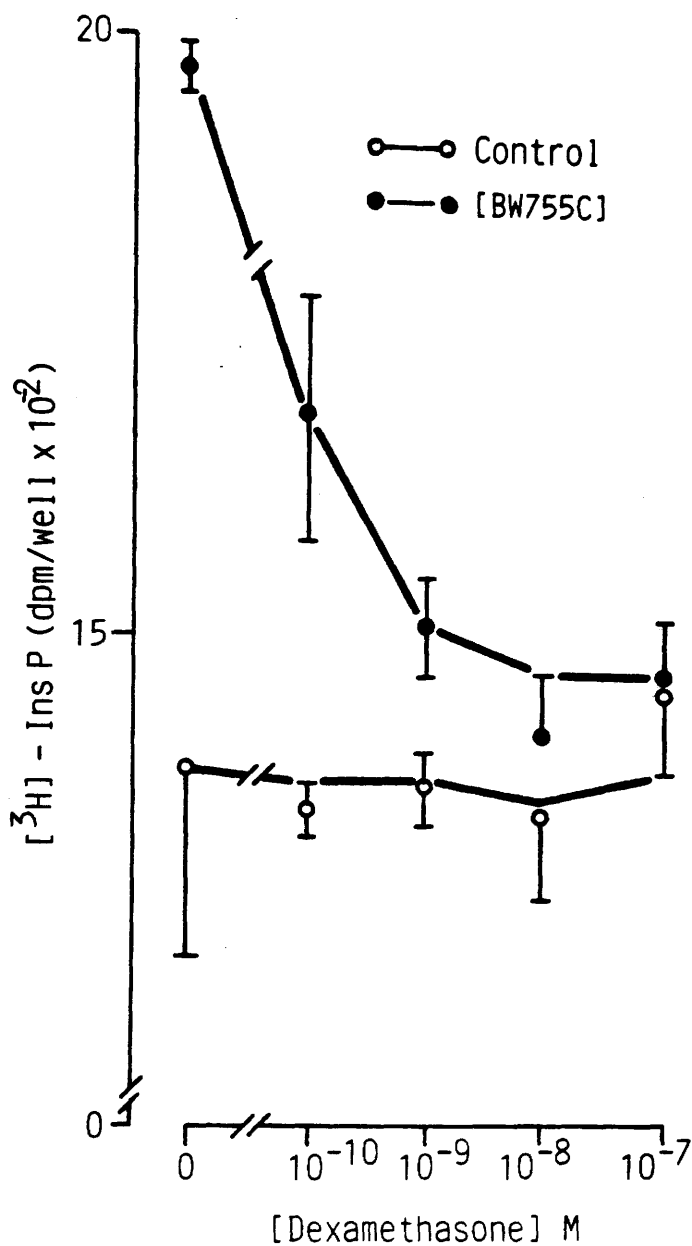


Figure 27: The effect of increasing concentrations of dexamethasone on the BW755C-induced rise in [³H]-InsP levels in Swiss 3T3 cells.

Cells were labelled to equilibrium with 5 μ Ci [³H]-inositol/well, and were treated with various concentrations of dexamethasone 24 hr prior to the experiment (control samples received ethanol vehicle). Samples were incubated for 10 min with 100 μ M BW755C. The above data are from a representative experiment and repeated once.

3.2.5 Studies using albumin-free balanced salt solution

Despite the evidence linking the presence of endogenous free AA with stimulated inositol lipid metabolism only rather unphysiologically high concentrations of exogenous AA (and other unsaturated fatty acids) stimulated InsP formation in 3T3 cells in full (albumin-containing) balanced salt solution (BSS). Similar data have recently been reported for a number of other systems, including blood platelets and placental cells (Hashimoto et al., 1985; Zeitler and Handwerger, 1985). However AA is known to bind to albumin avidly (Hoak et al., 1967) and it may be that the high levels of AA required to increase InsP formation in 3T3 cells are necessary because of its presence in the incubation mix at 0.1% (w/v).

3.2.5.1 The effects of AA in albumin-free BSS

In view of this, experiments were carried out to compare the effects of AA in 3T3 cells bathed in BSS containing 0.1% (w/v) BSA (bovine serum albumin), BSS containing 0.1% (w/v) fatty acid free BSA and BSS containing no albumin at all.

The results (Figure 28) quite clearly show that a concentration as low as 0.1 μ M AA gives a significantly greater increase in InsP levels where there is no albumin present in the bathing medium than where either albumin or fatty acid-free albumin is present. It is also noteworthy that where albumin is present, a concentration of 100 μ M AA is necessary to yield a similar rise in InsP, to that seen with 0.1 μ M AA, where there is no albumin present. Thus, in

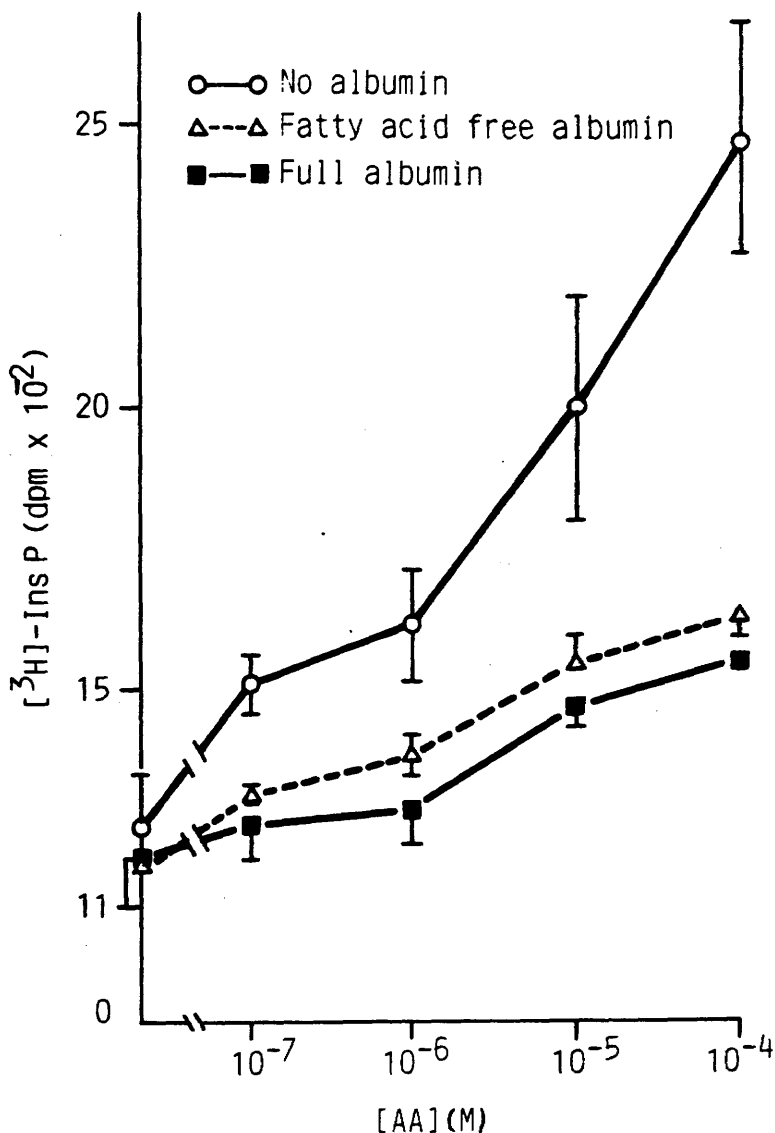


Figure 28: The effect of increasing concentrations of AA on $[^3\text{H}]$ -InsP levels in albumin-free Vs. albumin-present conditions, in Swiss 3T3 cells.

Cells were labelled to equilibrium with 5 μCi $[^3\text{H}]$ -inositol/well. Samples were either washed and left in albumin-free BSS, fatty acid free-albumin-containing BSS (0.1% (w/v)) or normal albumin-containing BSS (0.1% (w/v)). As in previous experiments 10 mM LiCl was used to amplify responses. Samples were then incubated for 10 min with various concentrations of AA. For other experimental details see Methods section. The above data are from a representative experiment carried out in triplicate and replicated once.

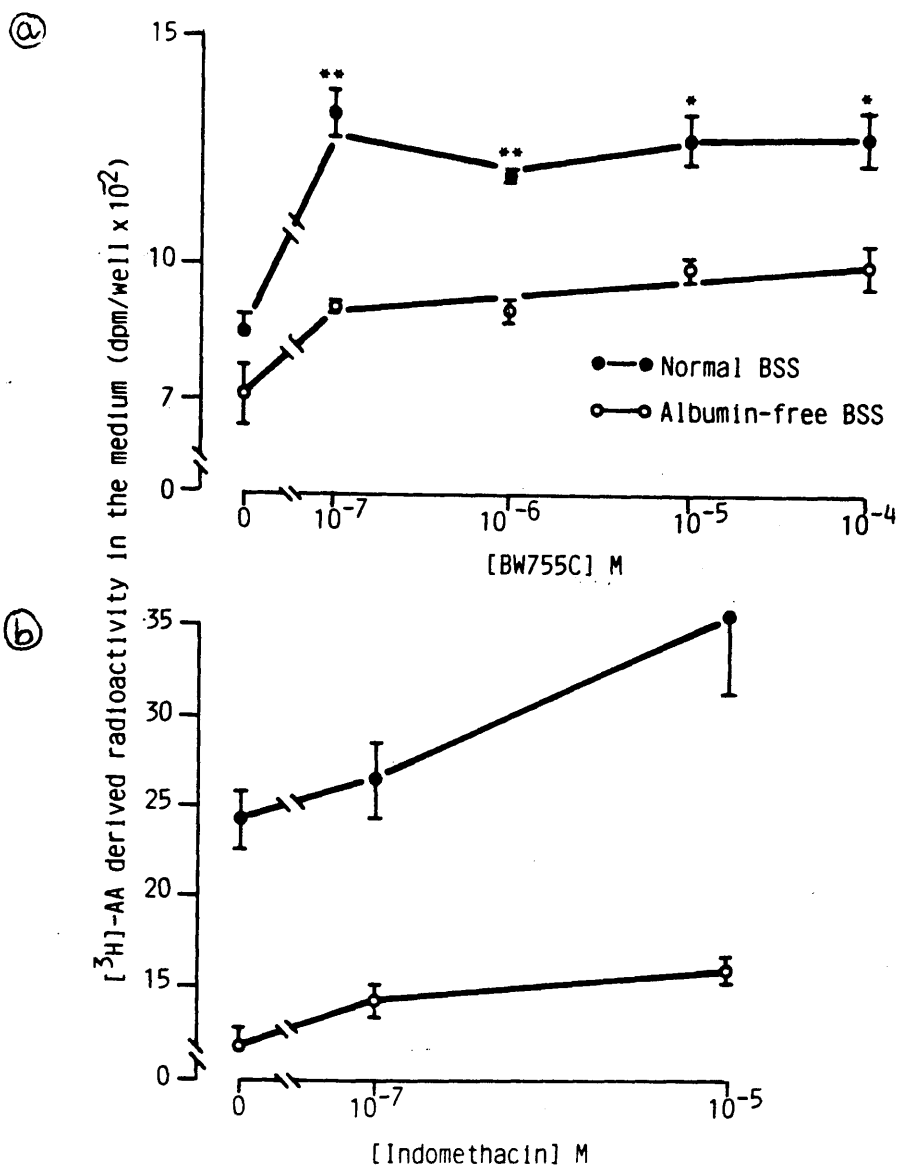


Figure 29: The effect of increasing concentrations of BW755C and indomethacin on [³H] -AA release from Swiss 3T3 cells in the presence and absence of albumin.

Cells were labelled to equilibrium with 0.5 μ Ci [³H] -AA/well. Half the samples were treated throughout the experiments with albumin-free BSS, while the other half were treated with (fatty acid free) albumin-containing BSS (0.1% (w/v)). Drug incubations were for 10 min.

(a) The data shown are from one experiment carried out in triplicate with BW755C.

(b) The data shown are from one experiment carried out in triplicate with indomethacin.

* $p < 0.05$

** $p < 0.001$

The data were subjected to a student's t-test to compare the values for cells in normal BSS with the values for cells in albumin-free BSS at a given concentration of BW755C

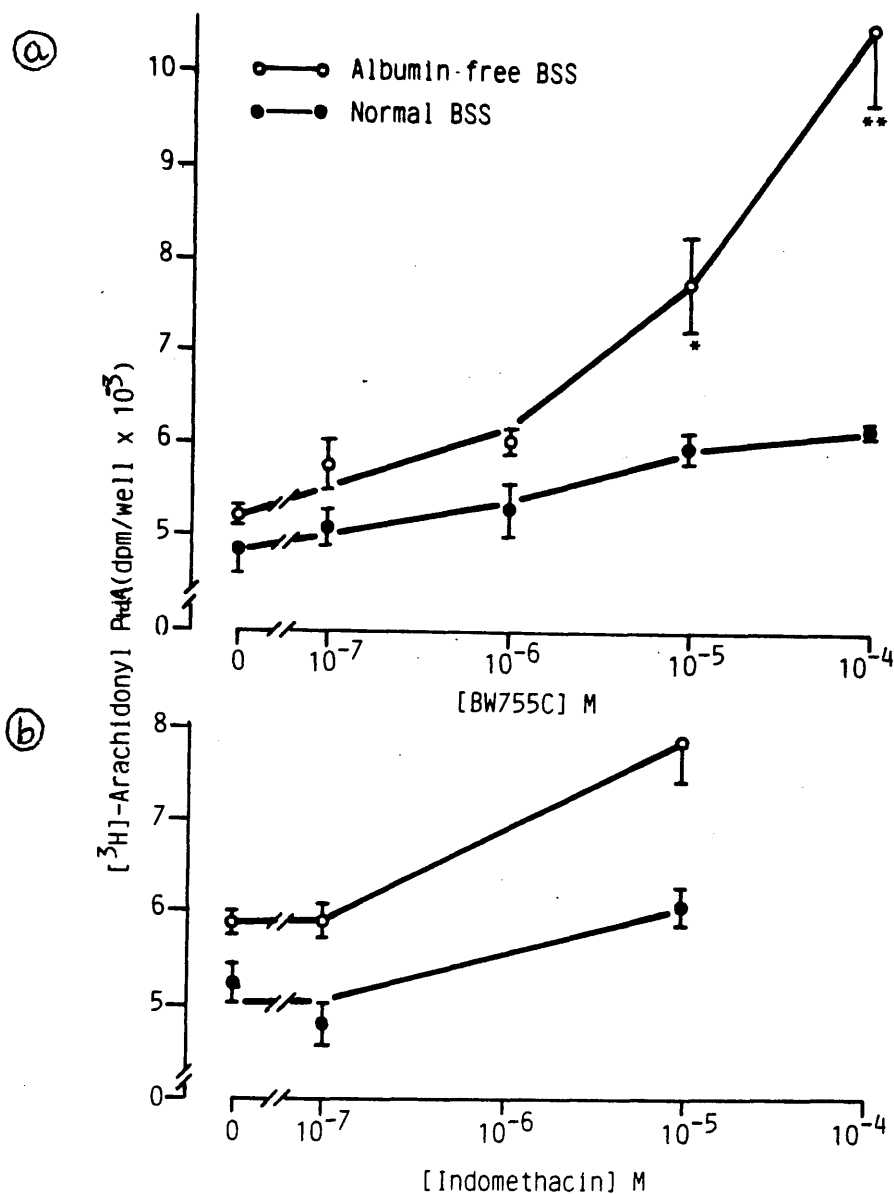


Figure 30: The effect of increasing concentrations of BW755C and indomethacin on [³H] -arachidonyl PtdA levels in Swiss 3T3 cells in the presence or absence of albumin.

Cells were labelled to equilibrium with 0.5 μ Ci [³H] -AA/well. Half the samples were treated throughout the experiments with albumin-free BSS, while the other half were treated with (fatty acid free) albumin-containing BSS (0.1% (w/v)). Drug incubations were for 10 min.

(a) The data shown are from one experiment carried out in triplicate with BW755C.

(b) The data shown are from one experiment carried out in triplicate with indomethacin.

The data were subjected to a student's t-test to compare the values for cells in normal BSS with the values for cells in albumin-free BSS, at a given concentration of BW755C

* $p < 0.05$ ** $p < 0.001$

In the case of indomethacin, also, the higher values for AA release are seen for cells surrounded by normal BSS (Figure 29b). However, here the effects of indomethacin are complicated by its ethanol vehicle. It is evident from Figure 29b that ethanol on its own causes a significantly greater increase in the release of AA than 0.9% saline (control). Thus, it is not possible to conclude here, that removal of albumin from the bathing medium leads to the changes in AA release observed.

The corresponding data for [^3H]-arachidonyl PtdA formation is shown in Figure 30 (a+b). It can be seen, here, that higher levels of arachidonyl PtdA formation, stimulated by both BW755C and indomethacin, are obtained from cells bathed in albumin-free BSS. However, again the effects of indomethacin are complicated by its ethanol control.

The results suggest that, as discussed above, the albumin binds AA outside the cell and prevents it from further interaction, either on the cell surface or within the cell. The absence of albumin allows a greater concentration of free AA, released into the bathing medium, to further interact on or within the cells, and thus leads to a greater increase in arachidonyl PtdA formation.

It is noteworthy, the degree of similarity between the graph shown in Figure 28 compared to that shown in Figure 30a. In Figure 28 the effect of increasing concentrations of exogenous AA on InsP levels in cells bathed in normal and albumin-free BSS, is illustrated. It can be seen that

increasing the concentration of BW755C yields increases in arachidonyl PtdA, i.e. increases in inositol lipid metabolism, which parallel those seen in Figure 28, due to increasing the exogenous concentration of AA. This might further suggest that the effects of BW755C, flurbiprofen and indomethacin on inositol lipid metabolism, are mediated by an accumulation of endogenous AA.

3.2.5.3 The effects of AA in combination with cyclooxygenase or lipoxygenase inhibitors in albumin-free BSS

If flurbiprofen, indomethacin and BW755C, stimulate the inositol lipid cycle by increasing the level of endogenous free AA, in 3T3 cells, then their effects on InsP would not be expected to be additive with exogenously applied AA. The data in Figure 31 show that this is indeed the case. There is no significant difference between InsP levels recorded in response to a submaximal concentration of AA (10 μ M) and that recorded in response to a combination of BW755C (100 μ M) and AA (10 μ M) or of flurbiprofen (100 μ M) and AA (10 μ M).

3.3 The effects of serum and PDGF on inositol lipid metabolism in Swiss 3T3 cells

The results from Section 3.2 suggested that arachidonic acid could potentially play a role in the mechanism whereby PDGF leads to the activation of inositol lipid metabolism. Indeed, it is known that PDGF can cause an increase in AA release (Shier & Trotter, 1980). However, the problem lies in proving the extent to which the effects of PDGF

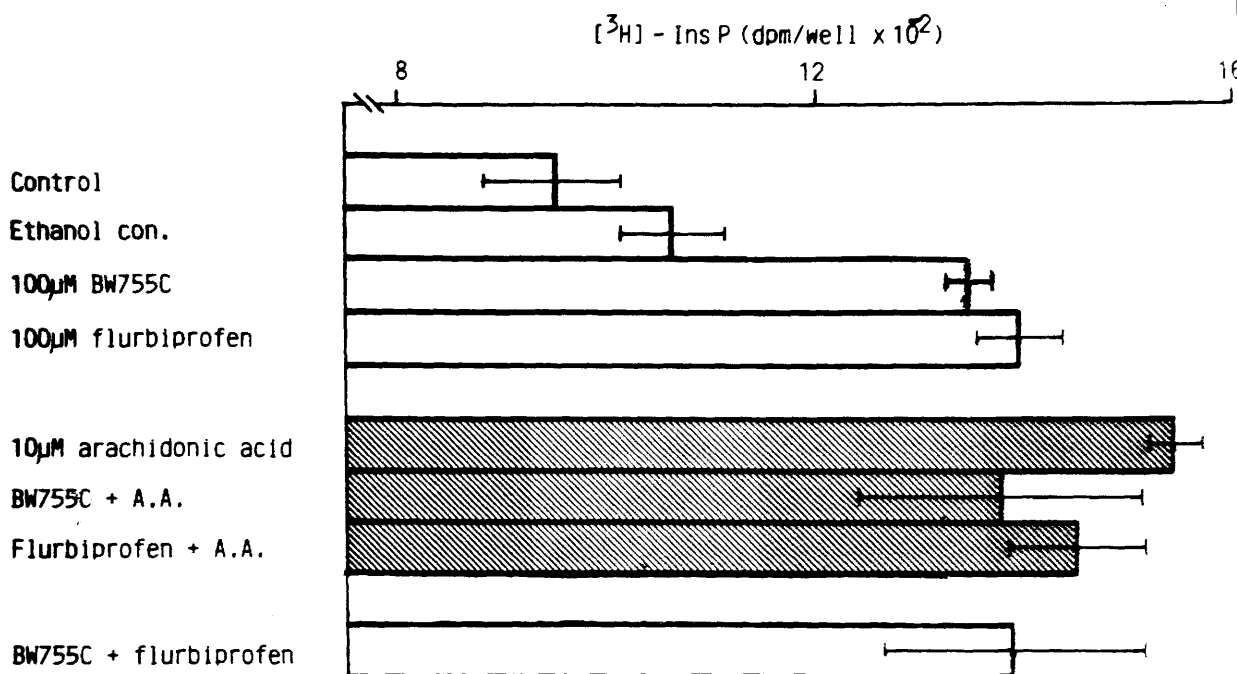


Figure 31: The effect of AA in combination with BW755C or flurbiprofen on $[^3\text{H}] - \text{InsP}$ levels in Swiss 3T3 cells in the absence of albumin.

Cells were labelled to equilibrium with 5 μCi $[^3\text{H}] - \text{inositol/well}$. The BSS contained 10 mM LiCl and no albumin. Samples were incubated for 15 min with the above drugs, except where combinations of drugs were added. In this case samples received 5 min. incubation with the c.o./l.o. inhibitor before addition of AA (for a further 10 min). In BW755C + flurbiprofen samples, the BW755C was added first for 5 min followed by 10 min with flurbiprofen. The data shown are from a representative experiment conducted in triplicate and repeated once.

on inositol lipid metabolism are due to stimulated AA release.

To tackle this problem, three strategies were devised. Firstly, in Section 3.2 it was shown that cyclooxygenase and lipoxygenase inhibitors caused increased inositol lipid metabolism and AA release. This suggested that the increase in AA release might be the cause of the increase in inositol lipid metabolism. Thus, if AA was involved in the action of PDGF on inositol lipid metabolism, a submaximal concentration of flurbiprofen or BW755C when combined with a submaximal concentration of PDGF, might increase its effects on inositol lipid metabolism in a synergistic manner.

Secondly, Murayama and Ui (1985) found that they could selectively block the increase in AA release, in thrombin-stimulated 3T3 cells, with pertussin toxin, leaving the increase in inositol lipid metabolism unaffected. Pertussis toxin interacts selectively with the guanine nucleotide regulatory protein N_i , that is involved in the receptor-mediated inhibitions of adenylate cyclase (see Section 1.1.5). It was therefore possible, that if a similar pertussis toxin-sensitive G-protein was involved in mediating the effects of PDGF on AA formation then its effects on inositol lipid metabolism might be completely abolished.

Lastly, it was also noted in Section 3.2 that the effects of flurbiprofen and BW755C on inositol lipid metabolism could be abolished, by pretreating the cells with dexamethasone. Thus, it might also be possible to inhibit, at least partially, the effects of PDGF on inositol

lipid metabolism by pretreating the cells with dexamethasone.

Unfortunately, it was not possible to use pure PDGF in all of the following experiments. Thus, dialysed newborn calf serum was used in the initial experiments, and, in the later experiments, the effects of highly purified PDGF, kindly donated by Dr Carl Heldin (see Methods), were examined.

Serum contains many growth factors and, therefore, it is most unlikely that the effects, observed in response to serum, are due entirely to PDGF. Thus, in an attempt to reduce this complexity, small molecular weight growth factors such as gastrin releasing peptide (a bombesin-like growth factor), $\text{PGF}_{2\alpha}$ and vasopressin, were removed by dialysing the serum using Micropor tubing, which has a molecular weight cut-off of 3,500 (see Methods). This left the large molecular weight factors such as PDGF and EGF in the serum sample (Besterman et al., 1986).

3.3.1 A comparison between non-dialysed and dialysed serum in stimulating inositol lipid metabolism in Swiss 3T3 cells

To show that the dialysis tubing was effective in removing small m.w. growth factors, the potency of non-dialysed serum in stimulating inositol lipid metabolism was compared with dialysed serum (Figure 32). Both batches of serum were from the same bottle, originally, and the non-dialysed serum was exposed to the same temperature conditions while dialysis of the second batch of serum was carried out. At all concentrations of serum used, the non-dialysed serum gave a significantly higher increase in InsP than the dialysed serum.

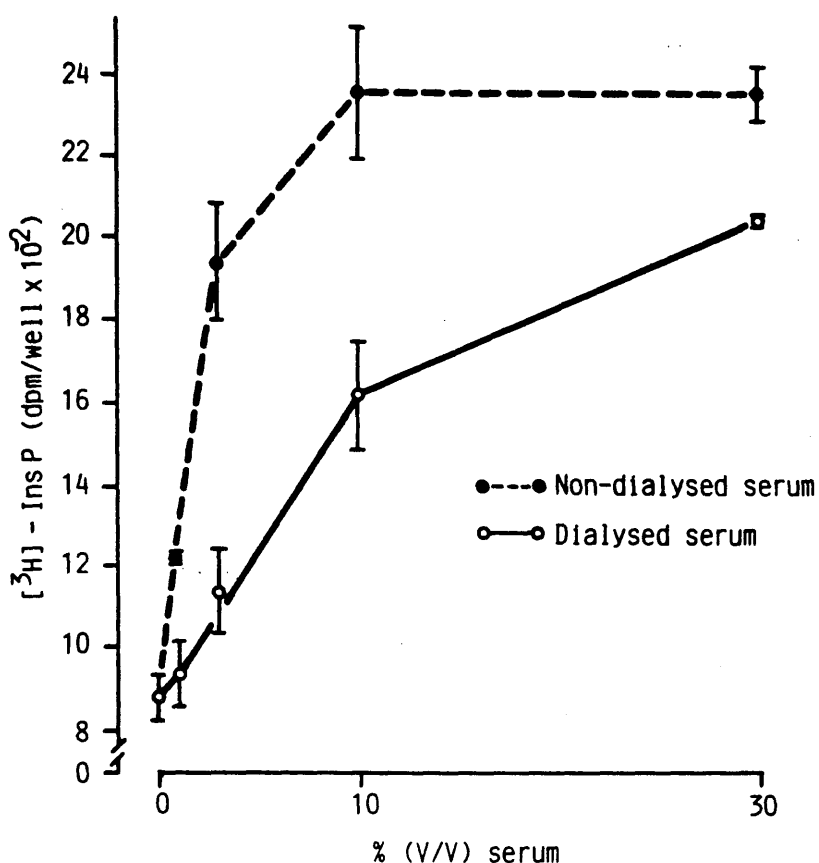


Figure 32: The effect of increasing concentrations of non-dialysed and dialysed newborn calf serum on $[^3\text{H}]$ - Ins P levels in Swiss 3T3 cells.

Cells were labelled to equilibrium with 5 μCi $[^3\text{H}]$ - inositol/well. Samples were incubated with various concentrations of serum (non-dialysed or dialysed) for 10 min. For other experimental details see Methods section (as in previous experiments 10 mM LiCl was present in the BSS). The data are from a representative experiment conducted in triplicate and repeated once.

The 3% (v/v) non-dialysed concentration showed the greatest difference, being equivalent to 30% (v/v) dialysed serum in its effect on InsP levels.

In the following experiments, dialysed new-born calf serum has been used, in order to relate the results more closely (although not absolutely) to those of PDGF.

3.3.1.1 The effects of dialysed serum in combination with BW755C on inositol lipid metabolism in Swiss 3T3 cells

From the previous experiments, it was clear that the largest increase in InsP levels was recorded with 30% (v/v) non-dialysed serum and a greater concentration of dialysed serum would presumably have been necessary to obtain this maximum increase in InsP levels. Now, if we are to believe that the release of free AA contributes to the action of PDGF on inositol lipid metabolism, then the effects of sub-maximal concentrations of serum might be increased by using drugs which increase the availability of AA. Unfortunately, these experiments, in which serum is combined with BW755C are complicated by the probable binding of AA to albumin and other proteins present in the serum.

In the experiment shown in Figure 33, the effects of increasing concentrations of BW755C combined with two sub-maximal concentrations of dialysed serum were examined. Dealing firstly with the 1.5% (v/v) concentration of dialysed serum (D.S.), it appears that submaximal concentrations of BW755C are not synergistic with the 1.5% (v/v) D.S., with regard to levels of InsP. However, at higher concentrations

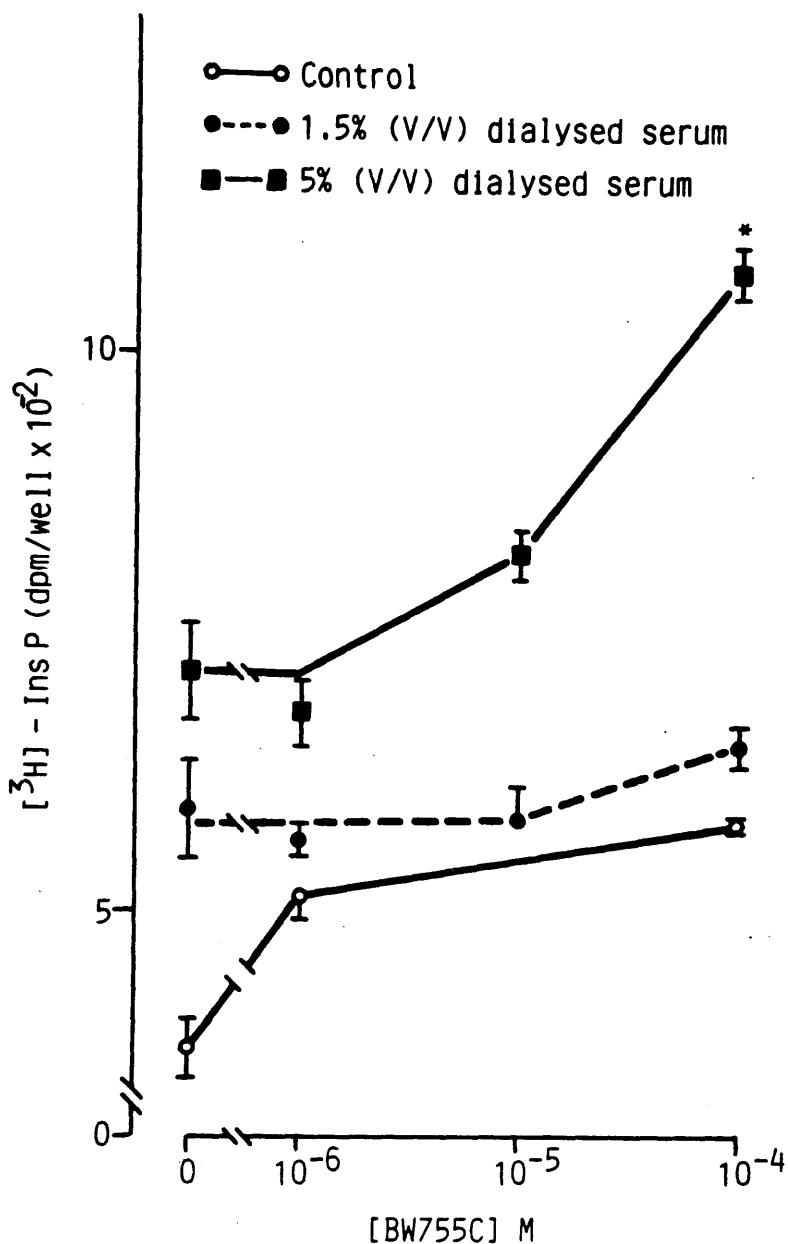


Figure 33: The effect of combining various concentrations of BW755C with two submaximal concentrations of dialysed serum on $[^3\text{H}] - \text{InsP}$ levels in Swiss 3T3 cells.

Cells were labelled to equilibrium with 5 μCi $[^3\text{H}] - \text{inositol/well}$. Samples received 5 min. incubations with various concentrations of BW755C prior to a further 10 min incubation with 5% (v/v) or 1.5% (v/v) dialysed serum. For other experimental details see Methods section. The above data are from a representative experiment conducted in triplicate and repeated once.

The data were subjected to a student's t-test, where the values of $[^3\text{H}] - \text{InsP}$ for dialysed serum-treated cells were compared with the values for control cells at each concentration of BW 755C.

* $p < 0.05$

of BW755C there appears to be a parallel rise in InsP in both the BW755C samples and the samples where 1.5% (v/v) D.S. is combined with BW755C. This effect is difficult to explain and will be covered in more detail in Section 4.

The results for 5% (v/v) D.S. in combination with BW755C are somewhat different. Again, low concentrations of BW755C are not synergistic with the D.S. in increasing InsP formation, but concentrations of BW755C above 1 μ M are additive with the serum. A synergistic effect results, when 100 μ M BW755C is combined with 5% (v/v) D.S. That is, the increase in InsP is significantly higher than 5% (v/v) D.S. on its own or than the projected additive value of 924 dpm/well [3 H] -InsP.

3.3.1.2 The effects of dialysed serum in combination with BW755C, on [3 H] -AA release and on [3 H] arachidonyl PtdA formation

If the synergy between D.S. and BW755C is to be believed, then it should also be observed when the release of AA and formation of arachidonyl PtdA are measured.

The results observed for AA release in response to D.S. and BW755C are shown in Figure 34. 1.5% (v/v) D.S. appears to be slightly inhibited when combined with a low concentration of BW755C (1 μ M). However, unlike the results for InsP formation, low concentrations of BW755C (1 μ M) significantly inhibit the increase in AA release in response to 5% (v/v) D.S. Concentrations of BW755C above 1 μ M are additive with 5% (v/v) D.S. Also, here, no synergy was apparent for

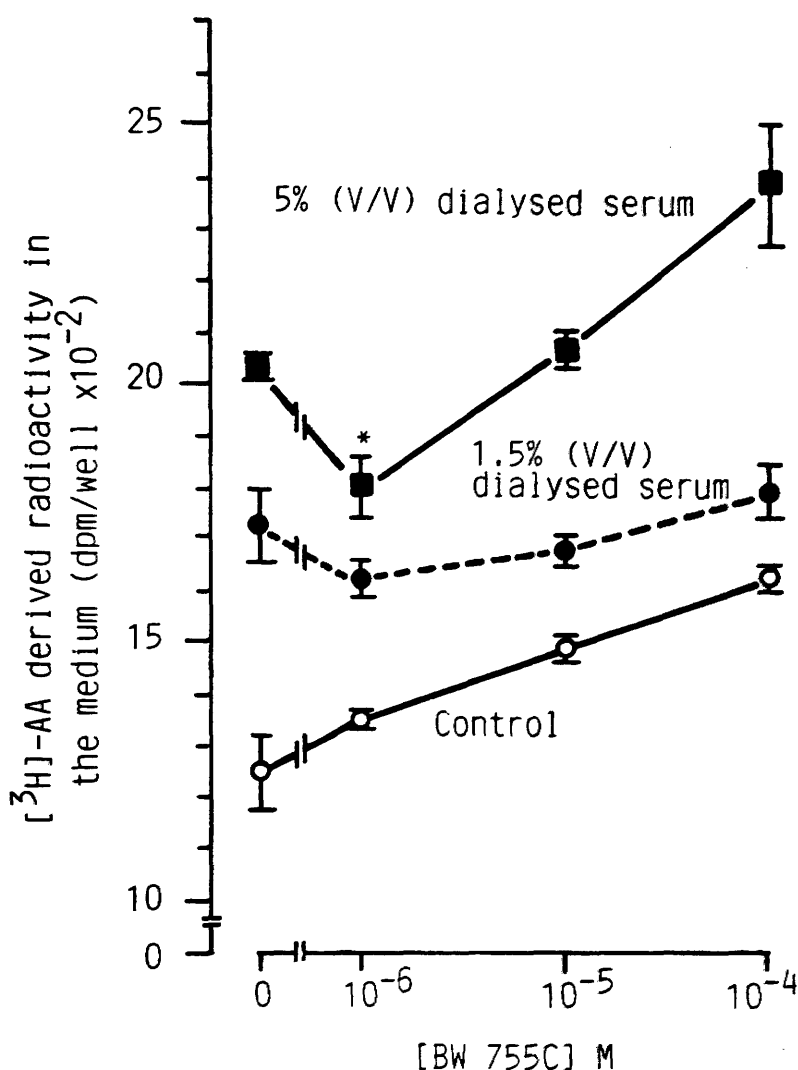


Figure 34: The effect of combining various concentrations of BW755C with two submaximal concentrations of dialysed serum on $[^3\text{H}]$ -AA release in Swiss 3T3 cells.

Cells were labelled to equilibrium with 0.5 μCi $[^3\text{H}]$ -AA/well. For experimental details see legend for Figure 33 and Methods section. The data shown above are from a representative experiment carried out in triplicate and replicated once.

The data were subjected to a Student's t-test, where the values for $[^3\text{H}]$ -AA released from dialysed serum-treated cells were compared with the values for $[^3\text{H}]$ -AA released from control cells at each concentration of BW755C

* $p < 0.05$

100 μ M BW755C and 5% (v/v) D.S., although the trend of the data might suggest that it occurs very slightly.

Turning to the data presented in Figure 35 we can examine the effects of combining BW755C and D.S. on arachidonyl PtdA formation. Similar results were obtained here as for InsP formation (Figure 33), except where 100 μ M BW755C was combined with 1.5% (v/v) D.S. In this case synergy was observed, not just additivity.

Unfortunately phospholipid extraction and analysis was not carried out in the first of the two experiments carried out here, and therefore the results shown in Figure 35 are based only on one experiment. Thus the experiment would have to be repeated, in order to show if this effect was reproducible.

Thus from the above data, it appears that BW755C, when combined with D.S. at certain concentrations can inhibit the effects of D.S., can have additive effects with D.S. or can synergise with D.S. to increase inositol lipid metabolism, AA release and arachidonyl PtdA formation. However, the interactions of the two drugs are far more complicated than might have been imagined. Certainly, at submaximal concentrations of the two agents, there is no evidence of the anticipated synergy that might result from the mechanism discussed above.

3.3.1.3 The effects of combining BW755C with other agonists on [3 H] -InsP formation in Swiss 3T3 cells

If BW755C could increase the effects of submaximal concentrations of D.S. on inositol lipid metabolism then, it could be argued, it might also increase the effects of

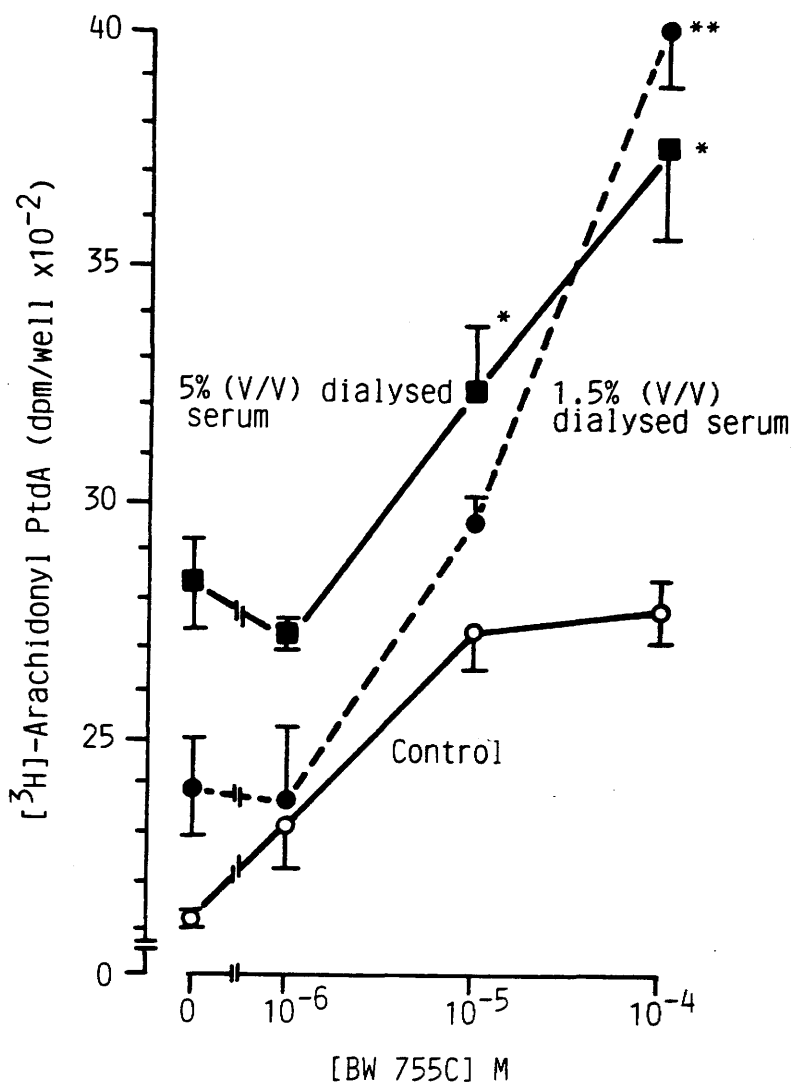


Figure 35: The effect of combining various concentrations of BW755C with two submaximal concentrations of dialysed serum on $[^3\text{H}]$ -arachidonyl PtdA levels in Swiss 3T3 cells.

As legend for Figure 34, except that the above data are from one experiment carried out in triplicate (but not repeated).

The data were subjected to a Student's *t*-test where values of $[^3\text{H}]$ -Arachidonyl PtdA from dialysed serum-treated cells were compared with the values of $[^3\text{H}]$ -Arachidonyl PtdA from control cells, at each concentration of BW755C

* $p < 0.05$ ** $p < 0.001$

other agonists. Figure 36 shows the effects of BW755C in combination with vasopressin, $\text{PGF}_{2\alpha}$, bombesin and 5% (v/v) D.S. on InsP formation. As in previous experiments, an enhancement of the effects of 5% (v/v) D.S. in InsP formation was observed, when combined with 100 μM BW755C. This high concentration of BW755C also significantly enhanced the effects on InsP observed with 1.6 μM $\text{PGF}_{2\alpha}$. It had no effect on the response to vasopressin or bombesin.

3.3.2 The effect of pertussis toxin on serum-stimulated responses in Swiss 3T3 cells

The second strategy incorporated to elucidate any involvement of AA in the action of PDGF on inositol lipid metabolism, was to abolish the agonist-induced increase in free AA formation with pertussis toxin. This idea, as discussed at the beginning of Section 3.3, arose from the findings of Murayama and Ui (1985). These authors abolished the rise in AA release from 3T3 cells, induced by thrombin, with pertussis toxin, but left the increase in inositol lipid metabolism unaffected. It was hoped that such a pertussis-sensitive G-protein might be involved in the effects which had been observed with BW755C, D.S., $\text{PGF}_{2\alpha}$ or bombesin. If such a G-protein could be inhibited then, any effects on inositol lipid metabolism observed with the above agonists, would not be as a result of increased AA release.

Therefore, cells were incubated overnight (18 hours) with 100 ng/ml pertussis toxin and then stimulated with various agonists for 10 min. The results (Figures 37 and 38)

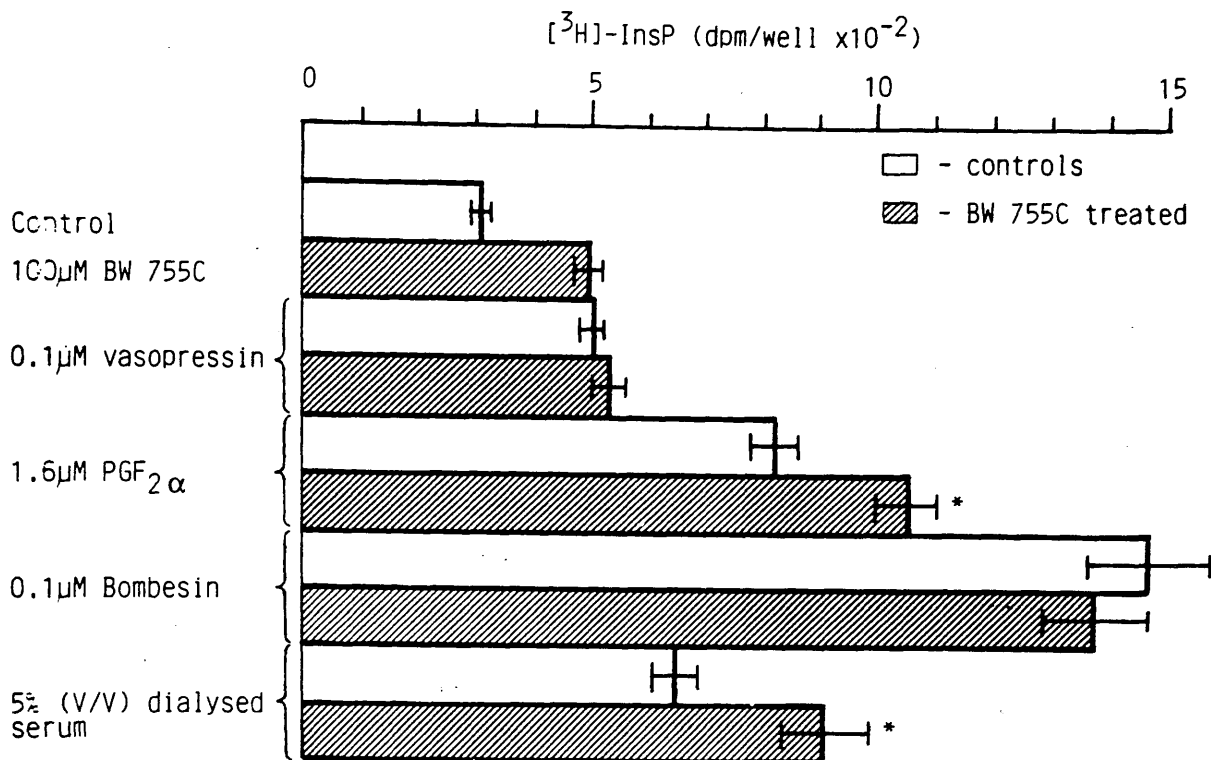


Figure 36: The effect of 100 μM BW755C on various agonist-induced increases in [³H] -InsP levels in Swiss 3T3 cells.

Cells were labelled to equilibrium with 5μCi [³H] -inositol/well. LiCl-containing (10 mM) BSS was used, and the samples were incubated for 5 min with 100 μM BW755C, prior to 10 min incubation with the above agonists. For other experimental details see Methods section. The data shown above are from an experiment carried out in triplicate and replicated once.

The data were subjected to a student's t-test, where the values of [³H]-InsP in BW755C-treated cells were compared to the values of [³H]-InsP in untreated cells, for each of the above agonists.

* p < 0.05

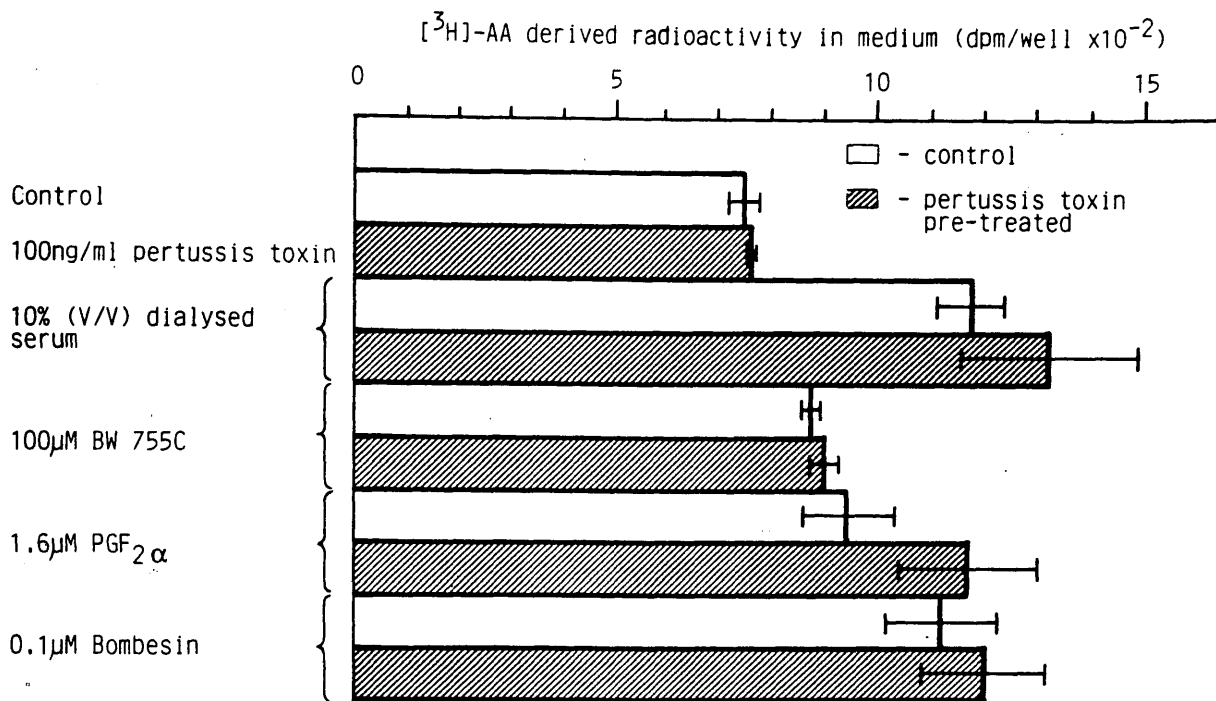


Figure 37: The effect of pertussis toxin on agonist-induced increases in $[^3\text{H}]\text{-AA}$ release from Swiss 3T3 cells.

Cells were labelled to equilibrium with 0.5 μCi $[^3\text{H}]\text{-AA}$ /well. Half the samples were incubated for 18 hr with 100 ng/ml pertussis toxin prior to the experiment where 10 min drug incubations were carried out. For other experimental details see Methods section. The above data are from one experiment carried out in triplicate.

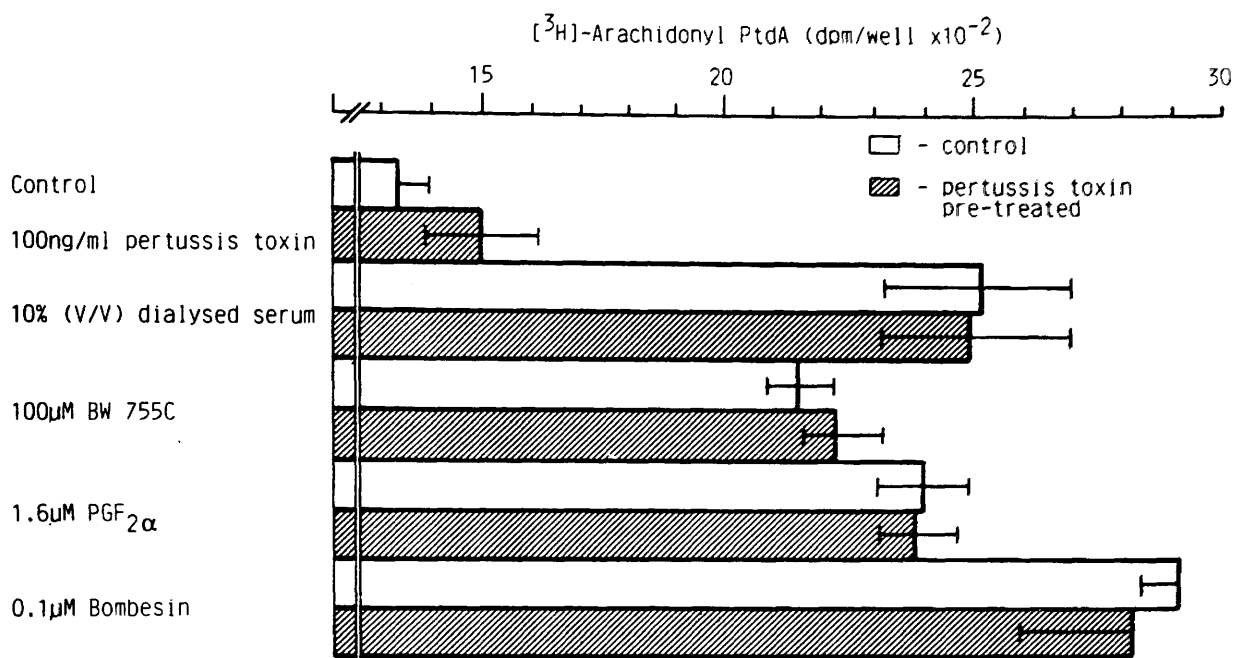


Figure 38: The effect of pertussis toxin on agonist-induced increases in [³H] -arachidonyl PtdA levels in Swiss 3T3 cells.

Cells were labelled to equilibrium with 0.5 µCi [³H] -AA/well. Half the samples were incubated for 18 hr with 100 ng/ml pertussis toxin prior to the experiment where 10 min drug incubations were carried out. For other experimental details see Methods section. The above data are from one experiment carried out in triplicate.

did not suggest the involvement of a pertussis toxin-sensitive G-protein. No significant difference in AA release or arachidonyl-PtdA formation was observed for any agonist in combination with pertussis toxin compared to the agonist on its own. These findings would therefore disagree with those of Murayama and Ui (1985). The pertussis toxin used in this experiment was kindly donated by Dr G. Milligan from a batch which, from his own experiments, was known to be active.

3.3.3 The effects of dexamethasone in combination with serum and PDGF on inositol lipid metabolism in Swiss 3T3 cells

Having failed to abolish agonist-induced AA release from 3T3 cells with pertussis toxin, an attempt was made to do this by a different method. In Section 3.2 it was seen that dexamethasone can abolish, in a dose-related manner, the effects of flurbiprofen and BW755C on inositol lipid metabolism. Dexamethasone is thought to act by indirectly inhibiting PLA₂ (via lipocortin) and thus prevent the formation of AA due to this enzyme (or group of enzymes).

In Sections 3.3.1.2 and 3.3.1.3 the results suggested that combining a high concentration of BW755C with 5% (v/v) D.S. (a submaximal concentration of D.S.) enhances the increase in inositol lipid metabolism observed for serum on its own. Thus, dexamethasone might reduce the effect of serum on inositol lipid metabolism, if the theory is correct.

3.3.3.1 The effect of dexamethasone in combination with serum on [^3H] -InsP formation

The data in Figure 39 show that, as in previous experiments, 0.1 μM dexamethasone abolishes the increase in InsP observed with 100 μM BW755C. Unfortunately, no significant reduction in the effects of 5% (v/v) D.S. or 10% (v/v) D.S. was observed with dexamethasone.

Dexamethasone did, however, reduce the effect of $\text{PGF}_{2\alpha}$ on inositol lipid metabolism, although it did not abolish it. Previously, it was noted, in Section 3.3.1.4, that the combination of BW755C with $\text{PGF}_{2\alpha}$ enhanced the increase in InsP observed for $\text{PGF}_{2\alpha}$ on its own. In combination these two pieces of evidence suggest that the effects of $\text{PGF}_{2\alpha}$ on inositol lipid metabolism in Swiss 3T3 cells, might be due, in part, to positive feedback on PLA_2 .

Bombesin remained unaffected by combination with dexamethasone, which would agree with the data shown in Section 3.3.1.4.

3.3.3.2 The effect of dexamethasone in combination with serum on [^3H] -AA release and on [^3H] -arachidonyl PtdA formation

The same general pattern of results seen in Section 3.3.3.1 appears when the effects of the agonists on AA release are examined, when they are combined with dexamethasone (0.1 μM).

Figure 40 shows the increase in AA recorded with BW755C is abolished in the presence of dexamethasone. Also dexamethasone does not significantly reduce AA release in

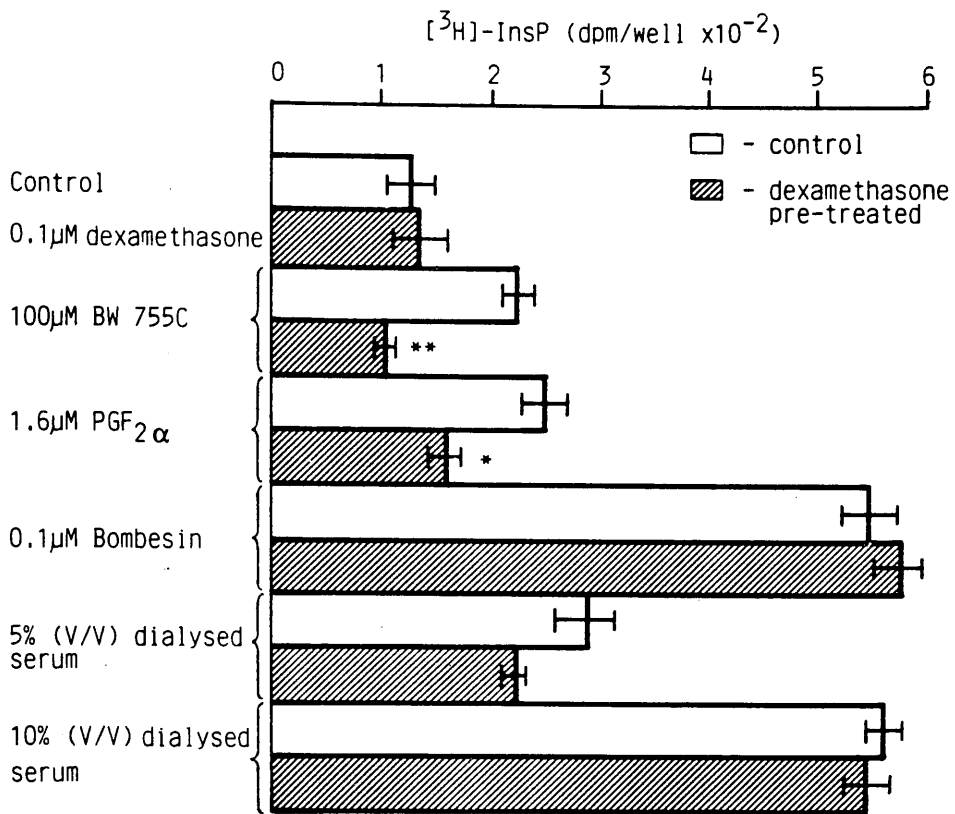


Figure 39: The effect of dexamethasone on agonist-induced rises in $[^3\text{H}]$ -InsP levels in Swiss 3T3 cells.

Cells were labelled to equilibrium with 5 μCi $[^3\text{H}]$ -inositol/well. Half the samples were incubated with 0.1 μM dexamethasone for 24 hr prior to the experiment. 10 mM LiCl-containing BSS was used throughout the experiment and drug incubations were for 10 min. The above data are from a representative experiment carried out in triplicate and repeated once.

The data were subjected to a Student's t-test where the values of $[^3\text{H}]$ -InsP obtained from dexamethasone-treated cells were compared with the values of $[^3\text{H}]$ -InsP obtained from untreated cells for each of the above agonists.

* $p < 0.05$ ** $p < 0.001$

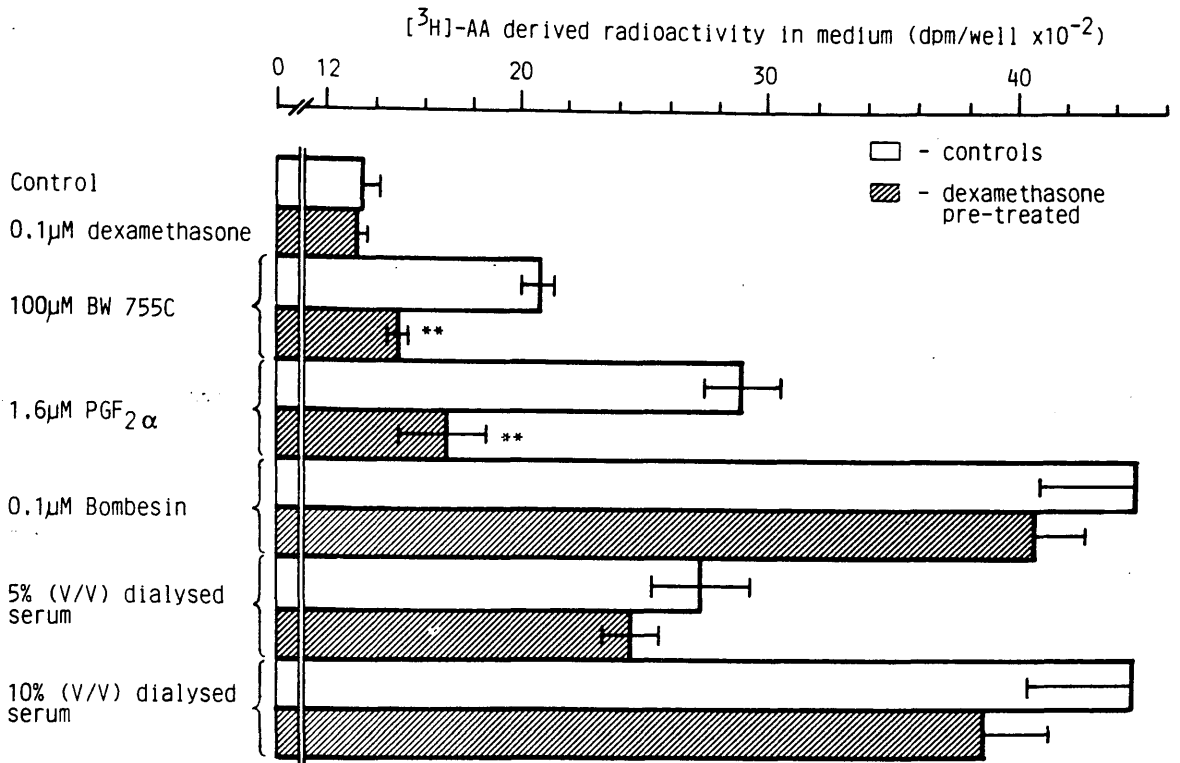


Figure 40: The effect of dexamethasone on agonist-induced rises in [³H]-AA release from Swiss 3T3 cells.

Cells were labelled to equilibrium with 0.5 μCi [³H]-AA/well. Half the samples were incubated with 0.1 μM dexamethasone for 24 hr prior to the experiment (control samples received an equal volume of ethanol vehicle). Drug incubations were for 10 min. The data shown above are from a representative experiment conducted in triplicate and replicated once.

The data were subjected to a Student's t-test where the values of [³H]-AA release obtained from dexamethasone-treated cells were compared with the values of [³H]-AA release obtained from untreated cells, for each of the above agonists.

** p < 0.001

response to D.S. (5% (v/v) or 10% (v/v)). AA release in response to $\text{PGF}_{2\alpha}$ is, as before, significantly reduced, but not abolished, by dexamethasone. The response to bombesin, once again, remained unaffected by dexamethasone treatment.

The effects of the agonists combined with dexamethasone on arachidonyl PtdA formation (Figure 41) are, for the most part, similar to those seen for InsP levels and AA release. The one exception is that in one of two experiments dexamethasone significantly reduced the effect of 10% (v/v) D.S. on arachidonyl PtdA formation. However, this was not observed in the second experiment and so it was not possible to reach a firm conclusion on any effect of dexamethasone on InsP formation, AA release or arachidonyl PtdA formation in response to D.S.

It was disappointing to find no significant reduction of the above serum-stimulated responses with dexamethasone. However, as we saw in Section 3.3.1, combining D.S. with other drugs can lead to far more complicated results than anticipated. The responses observed with D.S. may be due, in part, to other unidentified growth factors. Thus, it is not possible to directly relate the effects of dexamethasone on serum to PDGF. By using the highly purified PDGF it was hoped to reveal if this was the case. However, before this could be done, it was necessary to test the activity of the PDGF preparation.

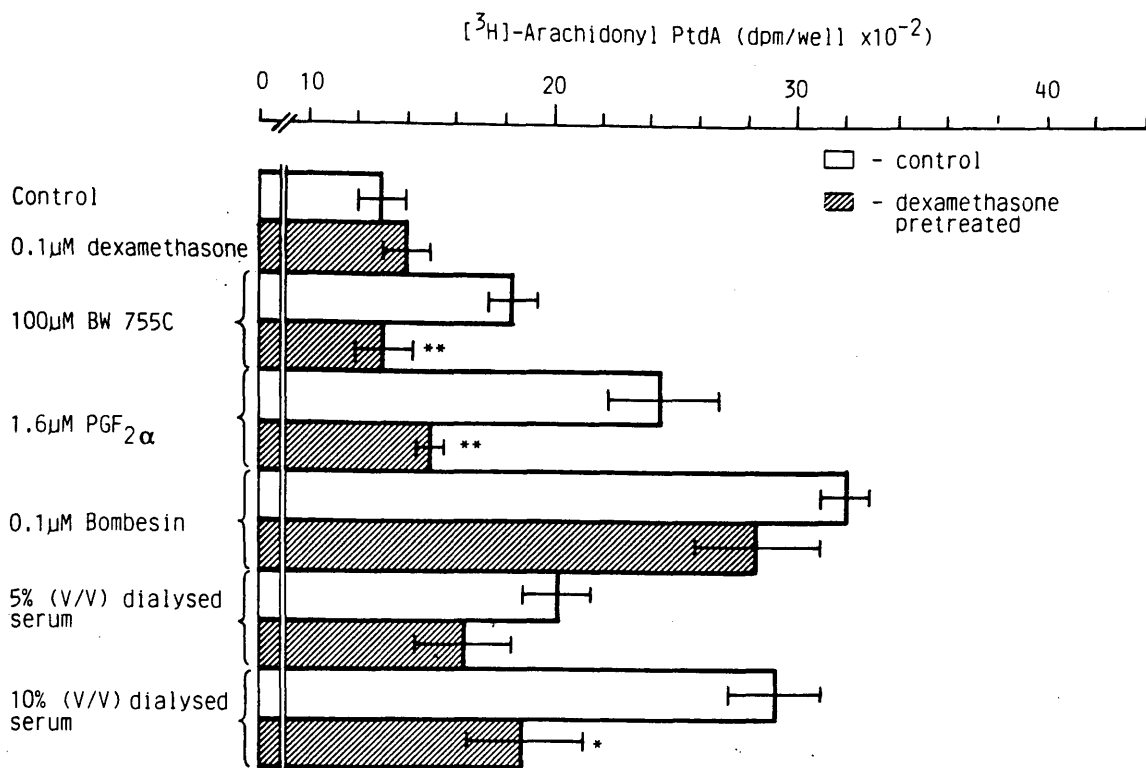


Figure 41: The effect of dexamethasone on agonist-induced rises in $[^3\text{H}]$ -arachidonyl PtdA levels in Swiss 3T3 cells.

Cells were labelled to equilibrium with 0.5 μCi $[^3\text{H}]$ -AA/well. Half the samples were incubated with 0.1 μM dexamethasone for 24 hr prior to the experiment (control samples received an equal volume of ethanol vehicle). Drug incubations were for 10 min. The data shown above are from a representative experiment conducted in triplicate and replicated once.

The data were subjected to a Student's t-test where the values of $[^3\text{H}]$ -Arachidonyl PtdA obtained from dexamethasone-treated cells were compared with the values of $[^3\text{H}]$ -Arachidonyl PtdA obtained from untreated cells, for each of the above agonists.

* $p < 0.05$ ** $p < 0.001$

3.3.3.3 The effect of PDGF on inositol lipid metabolism in Swiss 3T3 cells

Figure 42 shows the effects of 1 ng/ml and 10 ng/ml PDGF on [^3H] -InsP formation. 1 ng/ml PDGF gave a similar increase in InsP to that observed with 1.5% (v/v) D.S. (an increase of approximately 67% above basal). 10 ng/ml PDGF gave a 3-4 fold increase in InsP levels above basal. 1 nM bombesin gave a similar increase in InsP levels as 10 ng/ml PDGF and $\text{PGF}_{2\alpha}$ (1.6 μM), as noted before, caused about a doubling in InsP levels.

3.3.3.4 The effect of dexamethasone in combination with PDGF on inositol lipid metabolism

Knowing, now, that the highly purified PDGF was active in increasing inositol lipid metabolism, an experiment to investigate the effect of dexamethasone (0.1 μM) on (purified) PDGF-stimulated responses was carried out. Unfortunately, due to the availability of PDGF, this experiment was carried out only once. However, the results appear quite clear.

The data in Figure 43 reveal the results of this experiment. The effect of BW755C on [^3H] -InsP levels, as before, abolished by dexamethasone. Again, dexamethasone significantly reduced the response seen with $\text{PGF}_{2\alpha}$. The most important finding of this experiment was that the increase in InsP levels observed in response to 10 ng/ml PDGF was significantly reduced by dexamethasone (by approximately 20%).

Obviously, this experiment would have to be repeated before any firm conclusions could be arrived at. However, this experiment does suggest that AA might indeed have a role in mediating the action of PDGF on inositol lipid metabolism in Swiss 3T3 cells.

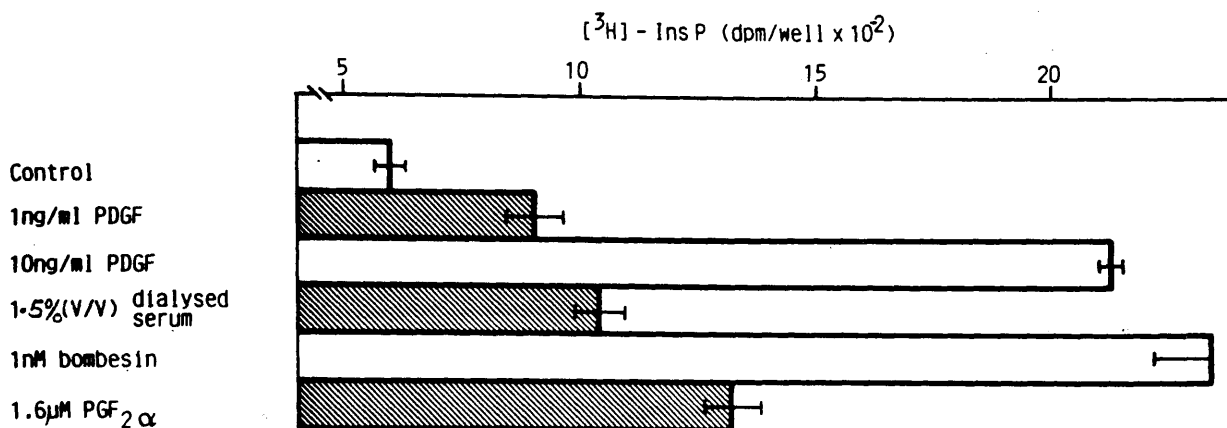


Figure 42: The effect of PDGF on [³H] -InsP levels in Swiss 3T3 cells.

Cells were labelled to equilibrium with 5 µCi [³H] -inositol/well. Samples were incubated with drugs for 10 min (for other experimental details see Methods section). The data shown above is from one experiment carried out in triplicate.

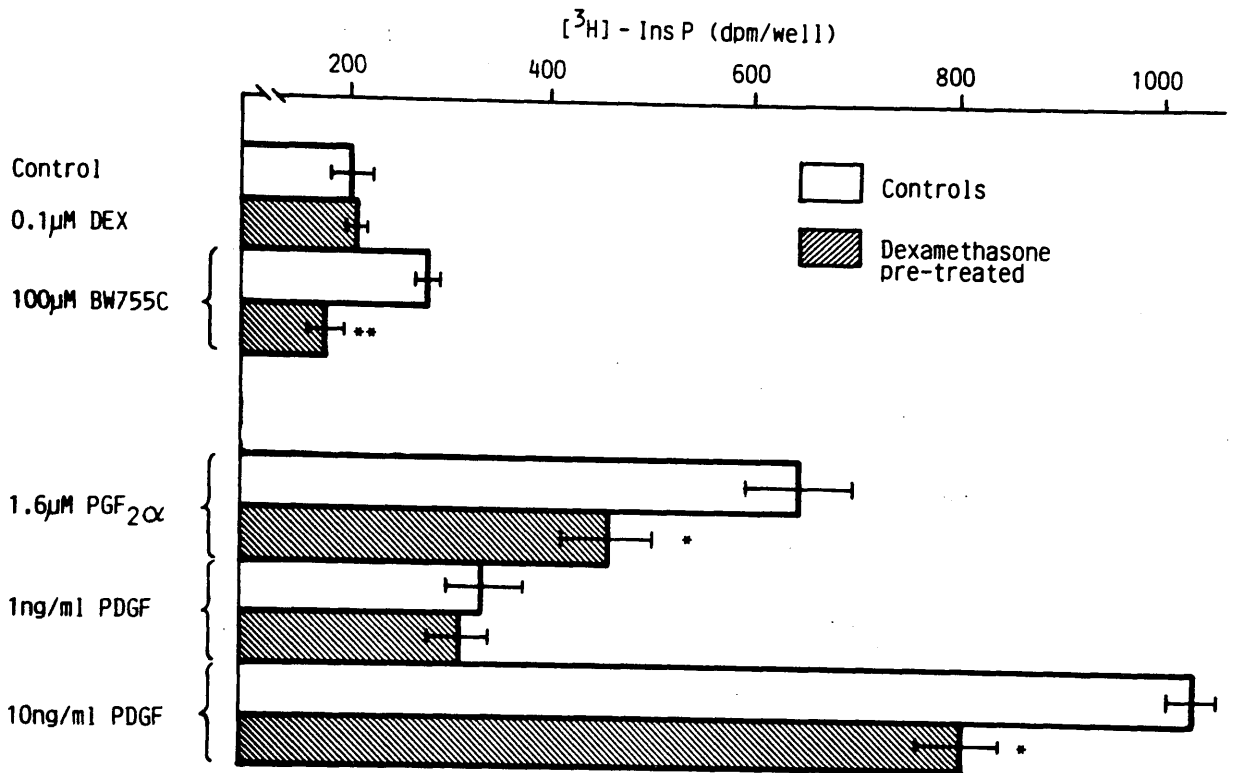


Figure 43: The effect of dexamethasone on PDGF-induced rises in $[^3\text{H}]$ -InsP levels in Swiss 3T3 cells.

Cells were labelled to equilibrium with 5 μCi $[^3\text{H}]$ -inositol/well. Half the samples were pre-incubated for 24 hr with 0.1 μM dexamethasone (the other half receiving ethanol vehicle). Drug incubations were for 10 min. The above data are from one experiment carried out in triplicate.

The data were subjected to a Student's t-test, where values of $[^3\text{H}]$ -InsP obtained from dexamethasone-treated cells were compared with values of $[^3\text{H}]$ -InsP obtained from untreated cells for each of the above agonists.

* $p < 0.05$ ** $p < 0.001$

4. DISCUSSION

4. Discussion

Enhanced inositol phospholipid metabolism in response to a Ca^{2+} -mobilizing agonist has been demonstrated in a number of different cells using a variety of agonists (Michell, 1975, 1979; Berridge, 1980; Putney, 1981).

As previously mentioned, (Section 1.1.4) the fatty acid on the 2-position of PtdIns (and the polyphosphoinositides) is mainly arachidonic acid, the precursor of a number of important molecules, such as the prostaglandins, prostacyclin, thromboxanes, leukotrienes and lipoxins. How arachidonic acid is released and to what extent each phospholipid contributes to the release has been the subject of many recent studies (see Irvine, 1982, for a recent review). The results have been divergent, but most investigators believe that AA is released by deacylation of diacylglycerols, formed by phosphodiesterase (phospholipase C) attack on PtdIns or its phosphorylated forms (Emilsson and Sundler, 1984), or by direct deacylation of phospholipids by phospholipase(s) of the A_2 type (Rittenhouse-Simmons, 1981; Billah and Lapetina, 1982). However the relative importance of these pathways in different cells and the mechanism of activation are still poorly understood.

The observation that receptors which induce changes in inositol lipid metabolism and produce a Ca^{2+} signal can also liberate AA, influenced Berridge (1981, 1982) to speculate that inositol lipid metabolism might represent a general transducing mechanism for generating a number of important intracellular signals. He noticed that activation

of Ca^{2+} -mobilizing receptors is almost invariably associated with the formation of prostaglandins and related metabolites.

Swiss 3T3 cells, utilized in this study produce prostaglandins (notably PGE_2 and $\text{PGF}_{2\alpha}$) from liberated AA. Because it begins very rapidly upon mitogen stimulation, release of AA and subsequent prostaglandin production has been suggested as a potential causal step in the mitogenic response (Fürstenberger and Marks, 1980). One of the released products, $\text{PGF}_{2\alpha}$, is itself a potent mitogen for 3T3 cells (Jimenez de Asua et al., 1975).

Studies with $\text{PGF}_{2\alpha}$ in Swiss 3T3 cells, in which measurements of PtdIns and 1,2 DG were made (Macphee et al., 1984), revealed that the prostaglandin caused an increase in inositol lipid metabolism. This was particularly interesting in the light of later experiments by a number of investigators, who showed that PDGF stimulated the breakdown of PtdInsP_2 to yield InsP_3 , thereby stimulating the release of intracellular free Ca^{2+} (Berridge et al., 1984; Moolenaar et al., 1984b) and also had a stimulatory effect on the metabolism of AA (Habenicht et al., 1981). Thus, our interest grew in investigating the possible role of AA and/or its metabolites in controlling inositol lipid metabolism in Swiss 3T3 cells and, in particular, whether such an action might link PDGF receptor activation with stimulated inositol lipid metabolism.

4.1 $\text{PGF}_{2\alpha}$ stimulates inositol lipid metabolism in Swiss 3T3 cells

At the outset of this study, it was known that $\text{PGF}_{2\alpha}$ was a mitogen for 3T3 cells and that it caused increased inositol lipid metabolism (Macphee et al., 1984). However,

a detailed study of the effects of $\text{PGF}_{2\alpha}$ on the individual metabolites of the inositol phospholipid cycle, and on the other major phospholipids of the plasma membrane was lacking. Thus, the initial intention of the project was to carry out such a study. In a broader sense, however, we also wished to investigate the role of AA and/or its metabolites in controlling inositol lipid metabolism in Swiss 3T3 cells.

To do this, cells were labelled with $[\text{}^3\text{H}]$ -inositol and in a series of experiments, the levels of various inositol metabolites were measured in response to $\text{PGF}_{2\alpha}$. It was found, as in many other cell types, for example GH_3 pituitary tumour cells, that by the inclusion of 10 mM LiCl in the balanced salt solution, the $\text{PGF}_{2\alpha}$ -stimulated increase in InsP levels could be amplified (Figure 6) (Hallcher and Sherman, 1980; Berridge et al., 1982; Drummond and Raeburn, 1984). In addition, an interesting finding from these experiments was that lithium did not have the same depleting effect on inositol levels as has been observed in certain other tissues, such as the adrenal gland (Sherman et al., 1986). This would suggest that Swiss 3T3 cells may contain a large pool of free inositol, which is consequently difficult to deplete. The resistance of the cellular inositol levels to lithium-induced depletion differs markedly from GH_3 cells (one of the best understood systems for this aspect). This raises the possibility that cellular diversity in respect of inositol depletion could contribute to the selective action of lithium on the central nervous system, if these cells were particularly prone to depletion (Drummond, 1987).

Also of interest, is the apparent inability of lithium to enhance the response to $\text{PGF}_{2\alpha}$ measured as increases in InsP_2 and InsP_3 content. An explanation of this might be that the enzymes that metabolize these substances, which, in any case, are notably less sensitive to lithium than the Ins-1-Pase(s) , might be working at low capacity and, therefore because Li^+ is an uncompetetive inhibitor (Hallcher and Sherman, 1980), be rather incompletely blocked.

HPLC analysis of the acid-soluble fractions in Swiss 3T3 cells, did not shed any great light on the above findings. It did reveal, however, that the majority of InsP present is the 1-phosphate isomer, although a little 4-phosphate can be detected. The major bisphosphate isomer appears to be the $\text{Ins}(1,4)\text{P}_2$ isomer: no $\text{Ins}(4,5)\text{P}_2$ and only a trace of what is presumably $\text{Ins}(3,4)\text{P}_2$ can be detected (Figure 14). These findings would be in agreement with Heslop et al. (1986) who reported the presence of a second InsP_2 other than $\text{Ins}(1,4)\text{P}_2$. The nature of the InsP_3 isomer(s) has not been fully clarified. Heslop et al. (1986) showed the presence of $\text{Ins}(1,4,5)\text{P}_3$ and $\text{Ins}(1,3,4)\text{P}_3$. Unfortunately, due to the small amount present in the cells and the age of the column used in this series of experiments we were unable to confirm this. If the $\text{Ins}(1,3,4)\text{P}_3$ isomer does exist, then presumably $\text{Ins}(1,3,4,5)\text{P}_4$ is also produced in 3T3 cells. Once again, as the data from the Dowex columns had indicated, no InsP_4 was detected. Obviously, further HPLC analysis will be necessary to ascertain which isomer(s) of InsP_3 are present in Swiss 3T3 cells and to confirm

the existence of InsP_4 in these cells, which has been found by Heslop et al. (1986).

The concentration of $\text{PGF}_{2\alpha}$ required to cause the maximum increase in inositol lipid metabolism is about $1 \mu\text{M}$ (Figure 7). Concentrations of $\text{PGF}_{2\alpha}$ as low as 1 nM can elicit a very small increase in inositol lipid metabolism (Figure 7), and this illustrates the potential importance of this mitogen. Hammarstrom (1977) has shown that up to 7.25 ng/ml $\text{PGF}_{2\alpha}$ could be released into the surrounding medium by 10^6 3T3 fibroblasts, in response to 50% (v/v) serum. Therefore in 2×10^5 cells (in each multiwell)/ 2 ml it can be calculated that 2.9 ng/ml $\text{PGF}_{2\alpha}$ could be produced. This is equivalent to $2 - 2.5 \text{ nM}$ $\text{PGF}_{2\alpha}$ in the surrounding medium. Thus, the finding that a concentration of $\text{PGF}_{2\alpha}$ as low as 1 nM is able to elicit a small increase in inositol lipid metabolism, suggests that it may be physiologically relevant.

The time course of the effects of $\text{PGF}_{2\alpha}$ on the metabolites of the inositol lipid cycle (Figures 8, 9, 10, 11, 12, 13) reveals that $\text{PGF}_{2\alpha}$ rapidly stimulates the turnover of inositol phospholipids. The separation of the polyphosphoinositides proved difficult by the method outlined by Jolles et al. (1981), due to the presence of an undefined substance which had a similar R_f value as PtdInsP on the T.L.C. plate. This is most likely lyso PtdIns , which migrates in this area. Thus, initially, only the total polyphosphoinositide content was measured (i.e. $\text{PtdInsP} + \text{PtdInsP}_2$, Figure 8). This revealed a rise in cellular polyphosphoinositide levels

within 40 seconds in response to $1.6 \mu\text{M}$ $\text{PGF}_{2\alpha}$. However, no initial decrease in these levels was detected, which might have been expected by analogy with the responses of other cell types to hormones (e.g. GH_3 cells, Macphee and Drummond, 1984). This decrease in PtdInsP and PtdInsP_2 occurs when hormone-stimulated hydrolysis of PtdInsP_2 is initiated, yielding InsP_3 and 1,2 DG. Breakdown of the lipid occurs rapidly, presumably before resynthesis of PtdInsP (and PtdInsP_2) can occur. Using the method of Creba et al. (1983) separation and measurement of the deacylated polyphosphoinositides was facilitated. Lyso- PtdIns , in this separation, would be eluted with Gro P Ins from the column and so would not contaminate Gro P InsP. Examination of shorter incubation periods with $\text{PGF}_{2\alpha}$ (Figure 9) shows that a slight decrease in PtdInsP and, to a lesser extent PtdInsP_2 , does occur in the first 10 seconds in $\text{PGF}_{2\alpha}$ -stimulated 3T3 cells. Levels of both polyphosphoinositides then increase rapidly above control values to a maximum within 1-2 minutes of $\text{PGF}_{2\alpha}$ administration. After this time, levels of the polyphosphoinositides begin to decline, but remain elevated above controls even after 20 minutes stimulation with $\text{PGF}_{2\alpha}$.

PtdIns levels are not significantly affected by $\text{PGF}_{2\alpha}$, although there is a suggestion from the data in Figure 9b that a decrease in PtdIns levels occurs at the time when the 3T3 cell polyphosphoinositide levels begin to increase. This would be in agreement with the findings of Macphee and Drummond (1984) in GH_3 cells, although in that system,

polyphosphoinositide levels are more clearly reduced at early time points.

From these results, it is no surprise that the effects of $\text{PGF}_{2\alpha}$ on the inositol phosphates is also very rapid (Figures 10, 11, 12, 13). InsP_3 levels are elevated to a maximum within 5 seconds of stimulation with $\text{PGF}_{2\alpha}$ (Figure 13c) and begin to decline back to basal after 20-30 seconds. InsP_2 levels do not rise quite as rapidly, reaching a maximum value after 20 seconds stimulation with $\text{PGF}_{2\alpha}$ (Figure 13b). InsP_2 levels decline very rapidly after this time. InsP levels reach a maximum value after 40 seconds stimulation with $\text{PGF}_{2\alpha}$ (Figure 13a). However, levels of InsP , although decreasing after 2 minutes, remain elevated above basal values for over 20 minutes, in cells stimulated with $\text{PGF}_{2\alpha}$ in the presence of 10 mM LiCl (Figure 10).

It is not clear whether this persistently elevated InsP level is sustained, as seems most likely, by a continual, although more modest, flux of metabolites through inositol phosphate metabolism, or whether it reflects complete inhibition, by lithium, of InsP degradation. The former view receives support from the fact that cellular PtdA levels are also persistently elevated in response to $\text{PGF}_{2\alpha}$ (Figure 15).

The time course of InsP accumulation is compatible with the view that InsP is not produced directly from PtdIns , but is formed by the hydrolysis of the InsP_3 . Nevertheless, it is difficult to be definitive on such aspects and in the light of contrary reports (e.g. Imai and Gershengorn, 1986), it might be wise to display some degree of circumspection.

From Table 5, it can be seen that $\text{PGF}_{2\alpha}$ (1.6 μM) causes a 2-3 fold increase in PtdA levels after 5 minutes incubation in Swiss 3T3 cells labelled with $[^3\text{H}]$ -glycerol. PtdIns, PtdS, PtdE and PtdCho levels are unaffected by $\text{PGF}_{2\alpha}$.

As in most cell types, the most abundant phospholipids of the plasma membrane appear to be PtdCho and PtdE. Similarly, the ratio of PtdIns to PtdS is about 1:1. The ratio of PtdA present in resting Swiss 3T3 cells compared with PtdIns is about 1:20.

It is interesting that a far greater response to $\text{PGF}_{2\alpha}$ is seen in PtdA formation compared with InsP formation where levels, at most, only double. Also, Macphee et al. (1984) only recorded an increase above basal of about 25% in 1,2 DG levels with 1.6 μM $\text{PGF}_{2\alpha}$. The differences in the levels of the above metabolites in response to $\text{PGF}_{2\alpha}$ might be best explained by variations in the kinetics of the enzymes responsible for PtdA formation, compared to those involved in InsP and 1,2 DG formation.

The time course of the effects of $\text{PGF}_{2\alpha}$ on PtdA levels is similar to that of InsP formation (Figure 15) PtdA formation in response to 1.6 μM $\text{PGF}_{2\alpha}$ is maximal within 40 seconds. In this case, however, levels of PtdA are maintained above basal over the 20 minutes stimulation period with $\text{PGF}_{2\alpha}$.

Thus, from these results, it would appear that $\text{PGF}_{2\alpha}$ in Swiss 3T3 cells, acts to stimulate the formation of the two second messengers of the inositol lipid cycle, namely, InsP_3 and 1,2 DG. Macphee et al. (1984) recorded an increase

in 1,2 DG in Swiss 3T3 cells in response to $\text{PGF}_{2\alpha}$ and here, it has been shown that PtdA levels rise. Morris et al. (1984) recorded an increase in Ca^{2+}_i after 10 seconds, in 3T3 cells, in response to $0.3 \mu\text{M}$ (100 ng/ml) $\text{PGF}_{2\alpha}$ which was maximal within 60-90 seconds. The above results show that in response to $1.0 \mu\text{M}$ $\text{PGF}_{2\alpha}$, InsP_3 levels rise to a maximum within 5 seconds and therefore the kinetics of the rise in $[\text{Ca}^{2+}]_i$ could be accounted for by the increase in InsP_3 levels.

The two second messengers of the inositol lipid cycle, as discussed in Section 1.1.4, have been implicated in cell proliferation (Berridge, 1984; Berridge and Irvine, 1984). Thus $\text{PGF}_{2\alpha}$ can be envisaged to act as a mitogen for Swiss 3T3 cells (Jimenez de Asua et al., 1981), by stimulating a rise in $[\text{Ca}^{2+}]_i$ via InsP_3 and by activating protein kinase C via 1,2 DG. Protein kinase C may directly or indirectly enhance Na^+/H^+ exchange resulting in increases in cytoplasmic Na^+ and pH (Dicker and Rozengurt, 1981; Schuldiner and Rozengurt, 1982; Cassel et al., 1983; Moolenaar et al., 1983; Rosoff et al., 1984).

The link between the formation of InsP_3 and DG, and the onset of cell proliferation is not understood. However, it has been shown that the expression of the c-fos proto-oncogene (see Section 1.2) is induced within a few minutes after exposure of fibroblasts to either PDGF or the tumour-promoting phorbol ester, phorbol 12-myristate 13-acetate), which is an activator of protein kinase C (Kruijer et al., 1984; Müller et al., 1984; Rabin et al., 1986).

The two second messengers, InsP_3 and DG, initiate a cascade of protein phosphorylation. However, only limited information is available on the phosphoproteins, and their activity, involved in this cascade and also on those phosphorylated by PDGF-dependent tyrosine kinase (Cooper et al., 1982; Ek and Heldin, 1984; Kohno, 1985). Therefore, it remains unknown how the inositol lipid turnover and the tyrosine kinase are connected to the induction of cell proliferation by PDGF.

It has been shown that $\text{PGF}_{2\alpha}$ and insulin have synergistic effects on cell proliferation in Swiss 3T3 cells (Jimenez de Asua et al., 1981). From the above data it can be seen that $\text{PGF}_{2\alpha}$ induces an increase in inositol lipid metabolism in Swiss 3T3 cells. Insulin activates tyrosine kinase (Kasuga et al., 1982a,b, and see Section 1.1.3) but does not induce inositol lipid metabolism (Macphee et al., 1984; Farese et al., 1984). Therefore, it is possible that inositol lipid metabolism-activated kinases and tyrosine kinase act synergistically in the initiation of cell proliferation.

An intracellular domain of the PDGF receptor has been shown to exhibit tyrosine kinase activity. However, the mechanism whereby PDGF stimulates inositol lipid metabolism remains elusive. The occupied PDGF receptor may act directly, to stimulate phospholipase C. It also seemed possible that PDGF may act by increasing the production of free AA which could be metabolized to active prostaglandins and/or leukotrienes (see Section 1.3 and Figure 5). One of the pieces of evidence which supports this idea is that $\text{PGF}_{2\alpha}$,

as we have seen from the above data, stimulates inositol lipid metabolism. Also, it has been shown that PDGF causes an increase in free AA levels in 3T3 cells (Habenicht et al., 1981).

Thus in order to show if the prostaglandins were intermediary in the action of PDGF on inositol lipid metabolism, it was necessary to be able to inhibit their formation.

4.2 Cyclooxygenase inhibitors stimulate inositol lipid metabolism in Swiss 3T3 cells

The results detailed in Section 3.2 are somewhat of a surprise (Figure 17). Flurbiprofen, a water-soluble inhibitor of cyclooxygenase (Figure 16) causes a 2-3 fold increase in PtdA formation in 3T3 cells. The effect is maximal with a concentration of flurbiprofen between 10 and 100 μ M (Figure 18). This increase in PtdA formation is comparable in magnitude to that seen with 1.6 μ M PGF_{2 α} . AA, itself, causes about a 2-fold rise in PtdA levels (Figure 17). Although the data is not shown, this effect is also seen with 10 μ M indomethacin.

To explain this unusual finding, it might be suggested that the flurbiprofen is not efficient in inhibiting cyclooxygenase, allowing a small amount of prostaglandin to be produced. Evidence that the c.o. inhibitors are effective, however, comes from studies where 3T3 cells are labelled with [³H] -AA. Flurbiprofen, in a dose-dependent manner, causes an increase in the level of free [³H] -AA in the medium surrounding the cells, in tandem with an

increase in the formation of [^3H] -arachidonyl PtdA (Table 6 and Figures 19 and 20). Indomethacin, likewise increases the level of [^3H] -AA in the medium and the formation of [^3H] -arachidonyl PtdA (Table 7).

The time course of flurbiprofen's effects on AA release and arachidonyl PtdA formation are outlined in Figures 19 and 20. AA release increases within 10 minutes of the addition of 30 μM flurbiprofen and continues to increase gradually between 10 and 40 minutes. The accumulation of arachidonyl PtdA peaks at 10 minutes and then declines, reaching basal levels within 25-40 minutes. From this it appears that the formation of arachidonyl PtdA and, to a lesser extent, the release of free AA, stimulated by flurbiprofen is relatively short-lived. One possible explanation for these data is that AA exerts a negative feedback effect on PLA_2 activity. Indeed, there is evidence (Ballou and Cheung, 1985) that, in human platelets, PLA_2 can be inhibited by unsaturated fatty acids. It is clear, however, that the transience of the effects of these drugs on inositol lipid metabolism contrasts with the data reported for PDGF (Hasegawa-Sasaki, 1985).

4.3 BW755C, an inhibitor of both c.o. and l.o. stimulates inositol lipid metabolism

Another possible explanation for flurbiprofen's effects on inositol lipid metabolism, could be that the flurbiprofen, by inhibiting c.o., redirects the AA towards the lipxygenase pathway (see Section 1.1.6). This effect has been seen in, for example, guinea-pig spleen, where indomethacin causes a

redirection of AA metabolism via the lipoxygenase pathway (Hamberg, 1976; see also Slepian et al., 1985).

Thus, a lipoxygenase metabolite might be produced which causes the increase in inositol lipid metabolism seen with flurbiprofen. The best way to test this idea, would be to block the lipoxygenase enzymes. Unfortunately, highly selective inhibitors of all the different l.o. enzymes (see Section 1.1.6) are not yet widely available. BW755C has, however, been reported to inhibit the 5, 12 and 15 lipoxygenases, in addition to c.o. (Higgs et al., 1979; Shoam and Razin, 1985; Needleman et al., 1986).

Data similar to that obtained with flurbiprofen are obtained with BW755C. This substance causes an increase in the level of free [^3H] -AA in the medium surrounding the Swiss 3T3 cells, and an increase in [^3H] -arachidonyl PtdA (Figures 21 and 22). These responses are maximal with a concentration of BW755C of 100 μM . This concentration of BW755C has been reported to cause the maximum inhibition of LTB_4 and TxB_2 synthesis by human polymorphonuclear leukocytes (Salmon, 1986). BW755C also increases the formation of InsP in a dose related-manner (Table 8).

One important point about all of the experiments in which flurbiprofen and BW755C were used, is the variability of the cells' responses. In 49 out of 57 experiments the drugs were effective at causing an increase in the level of InsP in Swiss 3T3 cells. However, in the remainder of experiments the drugs had no effect. As new preparations of drugs were used for each experiment it is unlikely that

the drugs' effectiveness had expired due to, for example, oxidation. One possibility could be that transformation of the cells might have occurred in areas on the petri-dish which were undetected, possibly leading to enzymatic changes in the cells. In the majority of experiments (86%), where the drugs were effective, the maximum response was also somewhat variable. For example, in one experiment the same concentration of flurbiprofen might cause a doubling in cellular InsP levels whereas in another experiment the increase might only be 60% above control values. This again might have resulted from the same conditions described above. It is worth noting, however, that if, as seems likely, the drugs are producing their effects by blocking arachidonate metabolism, then their effects will depend critically on the basal flux through the enzymes. In the light of this consideration, the variability becomes more readily explained.

The results obtained in the studies with BW755C make it appear unlikely that a stimulatory lipxygenase metabolite is the cause of the increase in inositol lipid metabolism seen with flurbiprofen although it remains a possibility that BW755C does not effectively block the production of all lipxygenase metabolites. This is an important point, for in the studies which measured AA and PG levels (Figure 23), it is possible that some hydroxyacids of AA, e.g. 15-HETE and 15-HPETE, might still be produced even in the presence of BW755C. These hydroxyacids would co-migrate with AA in the TLC separation used, and would, therefore, have been undetectable (Dr D.E. MacIntyre, personal communication).

Another possibility is that oxidation of AA by a cytochrome p450 enzyme could occur resulting in the formation of 19-hydroxy- and 19-oxo-eicosatetraenoic acid, 20-hydroxy-eicosatetraenoic acid and eicosatetraen-1, 20-dioic acids (Needleman et al., 1986). This has been shown to occur in rabbit renal cortex (Morrison and Pascoe, 1981; Oliw et al., 1987). Recently, a cyt-p450 enzyme capable of metabolizing AA has been localized to cells in the loop of Henlé of rabbit kidney (Ferrerri et al., 1984). Incubation of the cells with [^{14}C] -AA produced major products that were chromatographically different from known l.o. products and their synthesis was not inhibited by indomethacin, but was partially inhibited by SKF-525A and carbon monoxide; suggesting that they were produced by a cyt-p450-dependent reaction.

Thus, by inhibiting c.o. and l.o. enzymes with BW755C, AA could be redirected towards a cyt-p450-dependent enzyme in 3T3 cells, to produce a metabolite which, in principle, could be stimulatory in respect of inositol lipid metabolism. Such an enzyme has not been reported in these cells, however, a trace amount could theoretically exist.

The most logical conclusion from the data, is that the unsaturated fatty acid, itself, causes the increase in inositol lipid metabolism seen upon treatment of 3T3 cells with flurbiprofen or BW755C. These drugs would act to prevent the breakdown of AA and, thereby, allow it to accumulate. The persistence of elevated AA in the medium bathing the cells indicates, also, that reacylation reactions must be relatively slow in fibroblasts.

4.4 AA stimulates inositol lipid metabolism in Swiss 3T3 cells

The idea that AA could itself stimulate the inositol lipid cycle in cells has been suggested by other workers. For example Zeitler and Handwerger (1985) reported that in human placental cells, AA stimulated the rapid appearance of InsP. The effect of AA was specific for hydrolysis of phosphoinositides and phosphatidylserine and did not involve other phospholipids. Hashimoto et al. (1985) showed that high concentrations of AA (60 μ M) induced platelet aggregation and serotonin release that was not blocked by C.O. blockers. Such concentrations of AA also caused an increase in the levels of PtdA and 1,2 DG, although these increases were not thought to induce the platelet aggregation and serotonin release. Furthermore, AA has recently been reported to increase the intracellular Ca^{2+} concentration in rabbit neutrophils and several long chain fatty acids have been demonstrated to release Ca^{2+} sequestered by the sarcoplasmic reticulum of skeletal muscle (Cheah, 1981; Volpi et al., 1984). Wolf et al. (1986) showed that AA induced a rapid release of Ca^{2+} from isolated pancreatic islets, which was not due to the metabolites of AA. Comparison of AA-induced Ca^{2+} release with InsP_3 -induced Ca^{2+} release, revealed similar molar potency of AA and InsP.

Thus, because of the possibility that AA stimulated inositol lipid metabolism in Swiss 3T3 cells, the level of InsP was measured in response to various concentrations of AA (Figure 24). From these experiments, it appears that AA does, indeed, stimulate the inositol lipid cycle. However,

a high concentration of AA (100 μ M) is necessary to yield the maximum response.

The question arises, as to the physiological significance of this finding in the light of the need for such a high concentration of AA. One contributory factor, here, is that a large amount of the exogenously applied AA is likely to bind to albumin in the medium surrounding the cells (Hoak et al., 1967). This would be expected to markedly reduce the concentration of free (dissociated) AA which, presumably, is the active stimulant of inositol lipid metabolism.

To investigate this, the effect of AA on inositol lipid metabolism in 3T3 cells in albumin-free conditions was examined (Figure 28). It is evidence from these data that 0.1 μ M AA, under albumin-free conditions, gives an equivalent increase in InsP formation as 100 μ M AA in the presence of albumin (0.1% w/v). Further, in experiments where the effects of BW755C and indomethacin on AA release and on arachidonyl PtdA formation were studied, the results are consistent with the idea that endogenously released AA binds to albumin in the BSS surrounding the cells (Figures 29 and 30). Thus, [3 H] -AA release from cells appears greater when albumin is present in the surrounding BSS, due to it being "bound" outside the cell (Figure 29). [3 H] -Arachidonyl PtdA formation (Figure 30), on the other hand, is greater where the cells are in albumin-free BSS, as the endogenous AA released by the cell remains dissociated, thereby allowing it, presumably, to interact with, e.g., cell surface receptors.

These results could, in principle, be complicated by the possible binding of the drugs, themselves, to albumin. While it is likely that indomethacin will be bound by albumin, BW755C does not bind appreciably to albumin (Dr R. MacMillan, I.C.I., Cheshire, personal communication).

Unfortunately, the mass amount of AA contained in 3T3 cells has not been directly established. However, Habenicht et al. (1985b), have studied the effect of NaF and PDGF on AA release and PGE₂ formation in Swiss 3T3 cells. In response to 12 ng/ml PDGF, PGE₂ production rose by about 25 ng/10⁶ cells/ml of medium. From the data shown in Figure 23, about 95% of [³H] -AA derived radioactivity co-migrates with AA itself (in control and flurbiprofen-stimulated 3T3 cells). Thus the amount of PGE₂ present in 3T3 cells is equivalent to less than 5% of the AA content of the cells. Therefore if 25 ng/10⁶ cells/ml PGE₂ is formed in response to 12 ng/ml PDGF, then this might be equivalent to about 475 ng/10⁶ cells of free AA (in 1 ml medium). The multiwells, used in this project, contained about 2 x 10⁵ cells, and so the amount of AA, here, could be calculated to be about 95 ng/2 x 10⁵ cells. This is equivalent to 0.3 μM AA/2 x 10⁵ cells. Thus, the concentration of AA required to induce inositol lipid metabolism in 3T3 cells (in albumin-free conditions, Figure 28) of about 0.1 μM/2 x 10⁵ cells is close to the figure calculated for endogenous AA levels.

It is of interest that the mass of free AA released by 3T3 cells, at rest, is many times greater than the mass of the prostaglandin metabolites (e.g. PGE₂) of AA formed

under such conditions, assuming that the labelling employed, allowed full equilibration of [^3H] -AA with the different cellular pools of the fatty acid. This is consistent with the possibility that AA, itself, may play some mediator function that does not require its oxidation to a metabolite. Other investigators have also suggested this possibility based on the large amounts of unmetabolised arachidonate metabolites released in other stimulated systems (Hokin, 1985; Kolesnick and Gershengorn, 1985).

Also, consistent with this idea, is that if flurbiprofen and BW755C stimulate inositol lipid metabolism via an increase in endogenous free AA, in 3T3 cells, then their effects on InsP levels would not be expected to be additive with exogenously applied AA. The data shown in Figure 31 would support this.

Other unsaturated fatty acids, structurally related to AA can stimulate inositol lipid metabolism in 3T3 cells

If AA, itself, is a direct stimulant of inositol lipid metabolism, then the question arises as to its mechanism of action. A number of possibilities exist. For example, AA may bind to a receptor, which is coupled, via a G-protein, to phospholipase C. This receptor might be specific for AA, or the AA might bind to a "general" unsaturated fatty acid receptor. It is also possible that AA can bind to the receptor for $\text{PGF}_{2\alpha}$ that is thought to exist on these cells (Macphée et al., 1984). Another possibility is that AA could have a "fluidising" effect on the cell membrane

(Dave et al., 1981; Shier and Durkin, 1982). This might increase the mobility of the enzyme components within the cell membrane, e.g. a G-protein, such that they are in a better position to interact with the catalytic moiety, i.e. phospholipase C. Indeed, Dave et al. (1981) found that in membrane preparations of liver homogenates from C₃H mice, AA (4 µg/ml) stimulated an increase in ovine prolactin release of 73%, which they suggested was due to an increase in the number of receptor sites. The AA was also shown to decrease membrane microviscosity by 22%, which indicated that the membrane lipids became more fluid as a result of treatment with AA.

If a receptor existed which bound AA, then analogues of AA, or other unsaturated fatty acids might also stimulate responses in 3T3 cells, similar to those seen with AA itself. Dihomo- γ -linolenic acid and linoleic acid, unsaturated fatty acids related in structure to AA, do appear to possess some activity, although not as great as AA itself (Figure 25).

However, this does not clarify the mechanism of action of AA in stimulating inositol lipid metabolism, as these analogues could act at specific AA receptors, "general" unsaturated fatty acid receptors, PGF_{2 α} receptors or also have a fluidising effect on the cell membrane. Binding studies, with radiolabelled AA, could be carried out, which might reveal the presence of "receptors" on the plasma membrane. Fluidity of the membrane could be measured by fluorescence polarization of membrane preparations from Swiss 3T3 cells, treated with or without AA, using the

lipid probe 1,6-diphenylhexatriene, as outlined by Dave et al. (1981).

^{31}P -NMR-spectrum analysis of pure phospholipids, existing as hydrated bilayers, (e.g. pure yeast PtdCho), before and after addition of AA, might indicate a reorganisation of the tightly packed bilayer structure to one of an undefined nature (Dawson et al., 1984).

Regardless of the transduction pathway utilised by AA, in stimulating inositol lipid metabolism, in Swiss 3T3 cells, the mechanism whereby PDGF might stimulate the production of AA has yet to be established.

The theory proposed in Section 1.3 is that PDGF binding to its receptor, activates tyrosine kinase catalysis. This leads to the inhibition of p36 (lipocortin, an anti- PLA_2 protein), thereby freeing the PLA_2 enzyme(s) to hydrolyse membrane lipid and liberate free AA.

Recent work on p36 (Calpactin I, lipocortin II) has shown that it binds Ca^{2+} (Glenney, 1985, 1986a). Of particular interest, with respect to Ca^{2+} regulation, is that the affinity of p36 for Ca^{2+} can be greatly enhanced by anionic phospholipids in a manner similar to the effect of phospholipids and Ca^{2+} on protein kinase C activity (Glenney, 1985, 1986a, b). Studies by Wallner et al. (1986) indicated that pronounced sequence similarities existed between p36 and the human PLA_2 inhibitor lipocortin II. In the light of the finding that the calpactins I and II (p36 and p35) served as substrates for tyrosine kinase (Huang et al., 1986; Saris et al., 1986), these studies supported the theory outlined in Section 1.3.

However, some doubts have been raised about this theory. Saris et al. (1986) suggested that although p36 has been shown to inhibit porcine pancreatic PLA₂ with a specific activity similar to the inhibitory activity of lipocortin I (Huang et al., 1986), the possibility that the inhibitory activity of p36 is an incidental consequence of its ability to bind Ca²⁺ and phospholipid cannot be ruled out. Recent evidence from Davidson et al. (1987) indicated that this may be the case, as they reported that the most likely explanation for calpactin I (p36) inhibition of pancreatic PLA₂ in vitro is substrate depletion. If true, this would imply that the inhibition might not be specific for PLA₂ in terms of enzyme-inhibitor interactions. Further work is necessary to settle this issue. If PLA₂ is the most active enzyme in binding to these phospholipids, a specific blockade will still result.

Whichever way PLA₂ is activated, the result is the release of AA from membrane phospholipid. Hong and Deykin (1981) produced direct evidence, from measurements of lyso-PtdIns levels, for the in vivo activation of PLA₂ acting on PtdIns, in transformed BALB/c 3T3 cells.

Habenicht et al. (1981) showed that PDGF could stimulate the release of free AA in Swiss 3T3 cells. However, they suggested that AA is produced via the phospholipase C/diglyceride lipase pathway and they later showed that this pathway contributes, to some degree, to AA release in PDGF-stimulated Swiss 3T3 cells (Habenicht et al., 1985b).

In an effort to show that PLA₂ also contributed to PDGF-stimulated AA release in Swiss 3T3 cells, studies were carried out using the PLA₂ activator, melittin (Rozenfurt et al., 1981; Vincentini et al., 1984; Mix et al., 1984), and the indirect PLA₂ inhibitor, dexamethasone (Hirata, 1983; Dartois and Bouton, 1986).

4.5 Melittin stimulates AA release and arachidonyl PtdA formation in Swiss 3T3 cells

Melittin appears to stimulate a dose-related increase in both AA release and inositol lipid metabolism (seen as an increase in arachidonyl PtdA) in Swiss 3T3 cells (Table 9). Mix et al. (1984) have shown that melittin can induce a rise in intracellular free Ca²⁺ in human fibroblasts. This would be consistent with the theory that AA can control the inositol lipid cycle in fibroblasts, since melittin, by stimulating PLA₂ activity, would cause an increase in AA release and a subsequent fatty-acid dependent stimulation of inositol lipid metabolism. This would lead to an increase in Ins(1,4,5)P₃ formation, which as has been widely reported, (see Section 1.1.4), acts as a second messenger involved in [Ca²⁺]_i mobilization. Importantly, a rise in intracellular Ca²⁺ coupled with the addition of phorbol esters, (which are thought to stimulate protein kinase C), can lead to the activation of PLA₂ (Touqui et al., 1986). The protein kinase C is thought to act, here, by phosphorylating the anti-PLA₂ protein, lipocortin, and suppressing its anti-PLA₂ activity. Several long chain unsaturated fatty acids, including AA, have been demonstrated to release Ca²⁺ from intracellular

stores (e.g. Volpi et al., 1984) and AA, and other unsaturated fatty acids have been shown to activate protein kinase C. Kinase C activation by AA requires calcium and is enhanced by diolein, but does not require exogenous PtdS (McPhail et al., 1984). Thus, it may be possible that AA could act by stimulating both inositol lipid metabolism and PLA₂ activity. The PLA₂ activity may later decrease due to the AA having a negative feedback control on the enzyme (Ballou and Cheung, 1985).

4.6 Dexamethasone abolishes the increase in inositol phospholipid degradation observed with flurbiprofen and BW755C in Swiss 3T3 cells

Further data indicating the importance of the PLA₂ pathway, for the production of AA, are shown in Figures 26 and 27. In cells which were pre-incubated for 24 hours with 30 nM dexamethasone, 100 µM flurbiprofen does not induce a rise in InsP formation (Figure 26). Similar findings are obtained with BW755C (Figure 27). This effect of dexamethasone appears to be dose-related, as the increase in InsP formation observed with 100 µM BW755C can be reduced by a concentration of dexamethasone as low as 0.1 nM, and abolished with 10-100 nM dexamethasone. This is an important finding, as the low concentrations of dexamethasone necessary to reduce BW755C- and flurbiprofen-induced inositol lipid metabolism, are indicative of a specific glucocorticoid-receptor mediated action, perhaps on PLA₂ activity.

The phospholipase C/diglyceride lipase pathway may also be involved in the production of AA. However, since dexamethasone is so effective in abolishing the increase in inositol lipid metabolism, it would appear that the production of AA in 3T3 cells is dependent, to some extent, on PLA₂ activity. In order to test for the involvement of the PLC/DG lipase pathway, experiments could be carried out using the potent inhibitor of DG lipase, RHC 80267 (Chau and Tai, 1983). Unfortunately, neither the drug nor time was available to investigate this aspect further.

If released AA contributes to the effect of PDGF on inositol lipid metabolism. Then, by using dexamethasone to block production of AA, it might be possible, at least partially, to inhibit the effects of the growth factor in inositol lipid metabolism.

However, further strategies are necessary to prove the involvement of AA in PDGF action on the inositol lipid cycle. For example, it might be inferred that flurbiprofen and BW755C, by causing an accumulation of free AA, in Swiss 3T3 cells, and blocking its catabolism, could combine with a submaximal concentration of PDGF to increase inositol lipid metabolism, in an additive or, more likely, synergistic fashion. Synergy should occur since PDGF, by stimulating AA production and BW755C, by blocking AA metabolism, act by different mechanisms. However, since BW755C is active by itself, an additive interaction might result if the degree to which AA can stimulate inositol lipid metabolism is limited. For example, additivity or even mutual competition might

possibly be observed, if free AA at high concentrations, could exert a negative feedback control on the enzymes that release it from phospholipids. Such control has been described in human platelets (Ballou and Cheung, 1985).

A third approach, that might be useful in elucidating any mediatory involvement of AA (and its metabolites) in PDGF stimulation of the inositol lipid cycle, is derived from the work of Murayama and Ui (1985). They suggested that, in thrombin-stimulated 3T3 cells pertussis toxin could selectively block agonist-stimulated AA release, leaving the increase in inositol lipid metabolism unaffected. Thus, it is possible that if PDGF's effects on inositol lipid metabolism to PDGF, since PDGF is the major (but not the only) growth factor contained in serum (Rozengurt, 1980).

4.7 Dialysed newborn calf serum is less powerful than non-dialysed serum in stimulating inositol lipid metabolism in Swiss 3T3 cells

The ability of newborn calf serum to stimulate phosphoinositide degradation was compared with newborn calf serum, dialysed so as to retain peptide mitogens with a molecular weight greater than 3,500, e.g. PDGF, whilst removing lower molecular weight growth factors like GRP, $\text{PGF}_{2\alpha}$ and vasopressin.

Serum was dialysed using the method of Besterman et al. (1986). They found that in BALB/c 3T3 cells almost 70% of the capacity of foetal calf serum to stimulate phosphoinositide degradation was lost from dialysed serum compared to non-dialysed serum.

The data shown in Figure 32 indicates that the ability of newborn calf serum to phosphoinositide degradation is reduced by up to 90% when the serum is dialysed, and that 30% (v/v) dialysed serum gives an increase in InsP formation equivalent to that seen with about 3% (v/v) non-dialysed serum.

4.8 Pertussis toxin does not affect dialysed serum-stimulated responses in Swiss 3T3 cells

It might have been expected that pertussis toxin would inhibit serum-stimulated AA release, based on the results of Murayama and Ui (1985) and that arachidonyl PtdA formation would also be reduced if released AA exerted control on inositol lipid metabolism. Murayama and Ui (1985) suggested that, in thrombin-stimulated 3T3 cells, pertussis toxin inhibited a G-protein involved in the release of AA, but did not affect stimulated inositol lipid metabolism. Bokoch and Gilman (1984) also reported that pertussis toxin prevented FMLP receptor-mediated functions of neutrophils, including AA release, but the toxin's effects on inositol lipid metabolism were not studied.

The results of experiments in which 3T3 cells were pre-incubated for 18 hours with 100 ng/ml pertussis toxin (Figure 37) indicates that the toxin does not reduce AA release, in response to 10% (v/v) dialysed serum, 100 μ M BW755C, 1.6 μ M PGF_{2 α} or 0.1 μ M bombesin. Also, pertussis toxin has no effect on arachidonyl PtdA formation in response to the above agonists (Figure 38). This batch of toxin

was demonstrably active on the adenylate cyclase system, both prior and subsequent to this experiment (Dr G. Milligan, Glasgow University, personal communication), although not in this particular batch of 3T3 cells.

It is difficult to explain why these findings are so different from those described by Murayama and Ui (1985). Such differences may have resulted from variations in the experimental methods used in each case. Murayama and Ui pre-incubated their cells with pertussis toxin for only 3 hours prior to the experiment. In the data shown in Figures 37 and 38 the cells were pre-incubated for 18 hours prior to experimentation. It may be that a longer stimulation with pertussis toxin results in a change in the radio-labelling pattern of the phospholipids such that, upon stimulation with BW755C, a different pattern of [^3H] -AA release is observed. Also, Murayama and Ui pre-incubated their cells for 10 minutes with a buffer containing 50 μM AA (non-radioactive). This, they suggested, reduced the non-specific background of release during the subsequent release assay. Thus, in the data illustrated in Figure the AA release may be non-specific, arising possibly from degradation of DG by the PLC/DG lipase pathway, and not through the PLA_2 pathway. While it is also possible, that the pertussis toxin did not gain access to the G-proteins on 3T3 cells, the most likely implication is that AA release, seen here, is not mediated via a pertussis-sensitive G-protein. Indeed, Burch and Axelrod (1987) found that, in bradykinin-stimulated 3T3 cells, pertussis toxin did not affect PGE_2 synthesis or InsP formation.

4.9 BW 755C can have synergistic effects with submaximal concentrations of dialysed serum to increase inositol lipid metabolism in Swiss 3T3 cells

As discussed above, the most logical explanation of the effects of BW755C (and flurbiprofen) on inositol lipid metabolism in Swiss 3T3 cells, is that BW755C leads to an accumulation of free AA which, itself, stimulates inositol lipid metabolism. Thus, if AA is intermediary in the effects of PDGF on the inositol lipid cycle, then, the increase in inositol lipid metabolism, in response to submaximal concentrations of PDGF or, as in this case to dialysed serum, might be enhanced with BW755C.

The results from experiments designed to test this indicate that a more complicated situation exists in 3T3 cells. It appears that a submaximal concentration of BW755C can be inhibitory or additive with a submaximal concentration of dialysed newborn calf serum in increasing inositol lipid metabolism and AA release (Figures 33, 34 and 35). However, when a maximal concentration of BW755C is used, the results are different. Here, synergism between 100 μ M BW755C occurs with a submaximal concentration of dialysed serum. That is, the increases in inositol lipid metabolism and AA release, in response to a combination of 100 μ M BW755C and 5% (v/v) dialysed serum, are greater than the predicted additive increases from both agonists. A significant inhibitory effect of BW755C is only observed for serum-stimulated AA release (Figure 34). However, this effect appears to be

reproducible (in two experiments, carried out in triplicate). It is difficult to explain why such an inhibitory effect of a low concentration (submaximal) of BW755C on a submaximal concentration of dialysed serum occurs. In view of the complexity of the system under investigation it might be inappropriate to comment on this finding, without carrying out further investigation.

The additivity observed for the two growth factors, both at submaximal concentrations, might indicate that the AA-induced mechanism is not saturated by the drugs themselves, however, it might imply that the two agents act on different pathways in stimulating inositol lipid metabolism and AA release.

The synergistic effects observed with a maximal concentration of BW755C and a submaximal concentration of dialysed serum might suggest that the two drugs operate by different pathways to increase the concentration of free AA which, if our theory is correct stimulates inositol lipid metabolism. Based on our working hypothesis, we might have expected to observe synergism between BW755C and serum, although it is surprising that it does not occur at lower concentrations. A possible explanation of how synergism occurs, is that the agonists, by increasing AA production and accumulation, initially stimulate phosphoinositide degradation and, as a result, protein kinase C activation. Touqui et al. (1986) suggested that protein kinase C could phosphorylate the anti-PLA₂ protein, lipocortin, ultimately leading to production of AA. The increase in phosphoinositide

hydrolysis could also lead to increased $[Ca^{2+}]_i$, via $InsP_3$ formation, which could stimulate AA production via the Ca^{2+} -dependent PLA_2 enzyme. A positive feedback mechanism might exist for protein kinase C activation of PLA_2 . If production pathways for AA were saturated, using higher concentrations of the agonists, a threshold for inositol lipid metabolism might be reached whereby the positive feedback mechanism is activated and thus synergy could occur. The signal pathway through protein kinase C, is separate from and often synergistic to, the Ca^{2+} signalling pathway for the control of various cellular functions and proliferation (Nishizuka, 1986).

The results from these studies are inconclusive in that additivity and synergy could be explained in terms of the proposed theory that AA is involved in serum or PDGF-stimulated inositol lipid metabolism. Thus a more definitive experimental approach is necessary to demonstrate whether or not the theory is correct.

4.10 Dexamethasone can partially inhibit the serum or PDGF-induced increase in inositol lipid metabolism in Swiss 3T3 cells

If the additivity and synergy observed for BW755C and dialysed serum, in increasing inositol lipid metabolism, is at least consistent with the view that the agonists act through pathways which lead ultimately to an increase in free AA, then dexamethasone, which can abolish the BW755C-induced increase in inositol lipid metabolism, might also inhibit the serum-induced increase in inositol lipid metabolism.

Unfortunately, the data shown in Figures 39, 40 and 41 do not fit well with the above theory. That is, dexamethasone does not significantly reduce the effects of 5% (v/v) dialysed serum or 10% (v/v) dialysed serum on inositol lipid metabolism or AA release. Dexamethasone does, however, reduce the effect of $\text{PGF}_{2\alpha}$ on the inositol lipid cycle, although it does not abolish it. This is noteworthy, in light of the finding that BW755C enhanced the $\text{PGF}_{2\alpha}$ -induced increase in inositol lipid metabolism (Figure 36). In combination, these two pieces of evidence might suggest that part of $\text{PGF}_{2\alpha}$'s effects on inositol lipid metabolism, in Swiss 3T3 cells, might be due to stimulation of PLA_2 . Thus $\text{PGF}_{2\alpha}$ could act directly on its receptors to stimulate phosphoinositide hydrolysis. This would lead to protein kinase C activation and $[\text{Ca}^{2+}]_i$ mobilization. As explained above, PLA_2 activation could result from both of these responses and the subsequent release of AA might, by itself, stimulate inositol lipid metabolism, thereby contributing to the effect of $\text{PGF}_{2\alpha}$. Why a similar effect is not found with other agonists e.g. vasopressin, is unclear (Figure 36).

At the end of the time available for this project, it was possible to use highly purified PDGF (Figure 43). In this case, the results appear to be somewhat different. The PDGF-induced increase in inositol lipid metabolism (10 ng PDGF/ml) is significantly reduced by 0.1 μM dexamethasone. However, as indicated in Section 3.3.3.4, it was only possible to carry out this experiment once and therefore, it would be imperative to replicate these data in further experiments.

At the very most, however, it might be suggested that PDGF may act, in part, to stimulate the inositol lipid cycle, via activation of PLA_2 . This would lead to an increase in free AA formation which could stimulate inositol lipid metabolism directly, or be oxidated to, for example, $\text{PGF}_{2\alpha}$ which could also stimulate the inositol lipid cycle.

The fact that dexamethasone does not abolish the PDGF-induced increase in inositol lipid metabolism, might indicate the potency of the growth factor, in that PDGF is thought to stimulate a number of metabolic pathways which could interact with each other. This is more clearly illustrated in Figures 17, 28, 39 and 42, where the relative size of the inositol lipid response to PDGF is compared to that of other agonists. From the data shown in Figures 17, 28 and 39 it appears that 100 μM flurbiprofen, 1.6 μM $\text{PGF}_{2\alpha}$, 100 μM BW755C, 0.1 μM AA (in albumin-free conditions) and 0.1 μM vasopressin, all give a similar sized increase in inositol lipid metabolism. However 0.1 μM bombesin and 10 ng/ml PDGF are far more powerful than these agonists in increasing inositol lipid metabolism (Figures 39 and 42). Both bombesin and PDGF are thought to employ more than one signal transduction pathway in stimulating cell proliferation (Table 1).

Activation of the phospholipase C/diglyceride lipase pathway is also a potential route for PDGF action. This pathway would lead to the production of AA, but would not be inhibited by dexamethasone, and has been shown by Habenicht et al. (1985b) to be active in 3T3 cells. The

diglyceride lipase inhibitor RHC 80267 (Chau and Tai, 1983) could be used to determine the importance of this pathway in PDGF-induced inositol lipid metabolism in 3T3 cells.

4.11 Conclusions

This study has characterised $\text{PGF}_{2\alpha}$ -induced changes in inositol lipid metabolism in Swiss 3T3 cells. It has indicated that hydrolysis of inositol phospholipid is extremely rapid, occurring within 10 seconds of administration of $\text{PGF}_{2\alpha}$.

It has also suggested that the concentration of intra- or extracellular free AA may have a role in controlling inositol lipid metabolism. However, it remains possible that an as-yet-unidentified metabolite could also act to stimulate inositol lipid metabolism in Swiss 3T3 cells, although, the bulk of experimental evidence from this study would favour the former idea.

Further, this study has investigated whether the mechanism of action of PDGF, in leading to inositol phospholipid hydrolysis depends on an action of released free AA. This data, while clearly preliminary, might suggest that the action of PDGF on inositol lipid metabolism is mediated, in part, via AA production. However, the role of AA may be only minor.

The pathway of AA production has still to be resolved in these cells. While it appears that PLA_2 is active, as suggested by the potent inhibitory effects of dexamethasone, it is still possible that the phospholipase C/diglyceride lipase pathway also has a role in the production of AA. This latter area of study should prove an interesting avenue of research for the future.

5. REFERENCES

5. References

- Abrams, H.D., Rohrschneider, L.R., and Eisenman, R.N. (1982).
Cell 29, 427-439.
- Albert, P.R. and Tashjian, A.H. (1985). J. Biol. Chem. 260
8746-8759.
- Antoniades, H.N., Scher, C.D. and Stiles, C.D. (1979)
Proc. Natl. Acad. Sci. (U.S.A.) 76 1806-1813.
- Argiolas, A. and Pisano, J.J. (1983) J. Biol. Chem. 258
13697-13702.
- Armelin, H.A., Armelin, M.C.S., Kelly, K., Stewart, T.,
Leder, P., Cochran, B.H. and Stiles, C.D. (1984)
Nature (London) 310, 655-660.
- Ballou, L.R., and Cheung, W.Y. (1985) Proc. Natl. Acad. Sci.
(USA) 82 371-375.
- Batty, I.R., Nahorski, S.R. and Irvine, R.F. (1985)
Biochem. J. 232 211-215.
- Baud, L., Perez, J., Denis, M., Ardaillou, R. (1987)
J. Immunology 138 1190-1195.
- Becker, M.A., Dicker, P. and Rozengurt, E. (1983) Amer. J.
Physiol. (Cell Physiol) 244 C288-C296.
- Berridge, M.J. (1980) Trends. Pharmacol. Sci. 1 419-424.
- Berridge, M.J. (1981) Molec. Cell. Endocrinology 24 115-140.
- Berridge, M.J. (1982) in Calcium and Cell Function (W.Y.
Cheung, ed.) 3, 1-27. (Academic Press, New York).
- Berridge, M.J., Downes, C.P. and Hanley, M.R. (1982)
Biochem. J. 206 587-595.
- Berridge, M.J. (1983) Biochem. J. 212 849-858.
- Berridge, M.J. (1984) Biochem. J. 220 345-360.
- Berridge, M.J. and Irvine, R.F. (1984) Nature (London) 312
315-321.
- Berridge, M.J., Heslop, J.P., Irvine, R.F. and Brown, K.D.
(1984a) Biochem. J. 222, 195-201.
- Berridge, M.J., Buchan, P.B. and Heslop, J.P. (1984b)
Molec. Cell. Endocrinology 36, 37-42.

- Besterman, J.M., Watson, S.P. and Cuatrecasas, P. (1986)
J. Biol. Chem. 261 723-727.
- Billah, M.M. and Lapetina, E.G. (1982). J. Biol. Chem.
257 5196-5200.
- Bishop, J.M. (1983) Ann. Rev. Biochem. 52 301-354.
- Bishop, J.M. (1985) Cell 42 23-38.
- Blackwell, G.J., Flower, R.J., Nijkamp, F.P. and Vane, J.R.
(1978) Br. J. Pharmacology 62 79-89.
- Bokoch, G.M. and Gilman, A.G. (1984) Cell 39 301-308.
- Borgeat, P. and Samuelsson, B. (1979) Proc. Natl. Acad.
Sci. (USA) 76 2148-2152.
- Borgeat, P., Nadeau, M., Salari, H., Poubelle, P. and
Fruteau de Laclos, B. (1985) Adv. Lipid Res. 21 47-77.
- Bourne, H.R. (1986) Nature (London) 321 814-816.
- Brogden, R.N., Heel, R.C., Speight, T.M. and Avery, G.S.
(1979) Drugs 18 417-438.
- Brown, K.D., Blay, J., Irvine, R.F., Heslop, J.P. and
Berridge, M.J. (1984) Biochem. Biophys. Res. Commun.
123 377-384.
- Brown, K.D., Blakeley, D.M., Hamon, M.H., Laurie, M.S.,
and Corps, A.N. (1987) Biochem. J. 245 631-639.
- Brugge, J.S. and Erikson, R.L. (1977) Nature (London) 269
346-347.
- Brugge, J.S., Yonemoto, W. and Darrow, D. (1983) Molec.
Cell. Biol. 2 875-880.
- Burch, R.M. and Axelrod, J. (1987) Proc. Natl. Acad. Sci.
(USA) 84 6374-6378.
- Burgess, G.M., McKinney, J.S., Fabiato, A., Leslie, B.A.
and Putney, J.W. Jr. (1983) J. Biol. Chem. 258
15336-15345.
- Bürk, R.R. (1976) Exp. Cell Res. 101 293-298.
- Burns, C.P. and Rozengurt, E. (1983) Biochem. Biophys. Res.
Commun. 116 931-938.
- Butler-Gralla, E., Taplitz, S. and Herschman, R. (1983)
Biochem. Biophys. Res. Commun. 111 194-199.

- Carpenter, G., Lambach, K.J., Morrison, M.M. and Cohen, S.
(1975) J. Biol. Chem. 250 4297-4304.
- Carpenter, G. and Cohen, S. (1976) J. Cell Biol. 71 159-171.
- Carpenter, G. and Cohen, S. (1979) Ann. Rev. Biochem. 48
193-216.
- Cassel, D., Rothenberg, P., Zhuang, Y-Z, Deuel, T.F. and
Glaser, L. (1983) Proc. Natl. Acad. Sci. (USA) 80
6224-6228.
- Castagna, M., Takai, Y., Kaibuchi, K., San, K., Kikkawa, U.
and Nishizuka, Y. (1982) J. Biol. Chem. 257 7847-7851.
- Chau, L-Y, and Tai, H-H (1983) Biochem. Biophys. Res. Commun.
113 241-247.d
- Cheah, A.M. (1981) Biochim. Biophys. Acta 648 113-119.
- Claesson, H-K, Lindgren, J.A. and Hammarstrom, S. (1977)
FEBS Lett. 81 415-418.
- Cohen, S., Ushiro, H., Stoscheck, C.M. and Chinkers, M. (1982)
J. Biol. Chem. 257 1523-1531.
- Collins, M.L.K. and Rozengurt, E. (1982) J. Cell Physiol.
122 42-50.
- Cooper, J.A., Bowen-Pope, D.F., Raines, E., Ross, R. and
Hunter, T. (1982) Cell 31 263-273.
- Cooper, J.A. and Hunter, T. (1983) Curr. Top. Microbiol.
Immunol. 107 125-162.
- Cooper, J.A., Nakamura, K.D., Hunter, T. and Weber, M.J.
(1983a) J. Virology 46 15-28.
- Cooper, J.A., Reiss, N.A., Schwartz, R.J. and Hunter, T.
(1983b) Nature 302 218-223.
- Corvera, S. and Garcia-Sainz, J.A. (1984) Biochem. Biophys.
Res. Commun. 119 1128-1133.
- Courtneidge, S.A. and Bishop, J.M. (1982) Proc. Natl. Acad.
Sci. (USA) 70 7117-7121.
- Creba, J.A., Downes, C.P., Hawkins, P.T., Brewster, G.,
Michell, R.H. and Kirk, C.J. (1983) Biochem. J.
212 733-747.
- Czech, M.P. (1981) Amer. J. Med. 70 142-150.

- Czech, M.P., Massagué, J. and Pilch, P.F. (1981) Trends Biochem. Sci. 6 222-225.
- Dalla Favera, R., Bergni, M., Erikson, J., Patterson, D., Gallo, R. and Croce, C.M. (1982) Proc. Natl. Acad. Sci. (USA) 79 7824-7827.
- Dartois, E. and Bouton, M. (1986) Biochem. Biophys. Res. Commun. 138 323-329.
- Dave, J.R., Knazek, R.A. and Liu, S.C. (1981) Biochem. Biophys. Res. Commun. 103 727-738.
- Davidson, F.F., Dennis, E.A., Powell, M., Glenney, J.R. Jr. (1987) J. Biol. Chem. 262 1698-1705.
- Dawson, R.M.C., Irvine, R.F., Bray, J. and Quinn, P.J. (1984) Biochem. Biophys. Res. Commun. 125 836-842.
- Dicker, P. and Rozengurt, E. (1980) Nature (London) 287 607-612.
- Dicker, P., Pohjanpelto, P., Pettican, P. and Rozengurt, E. (1981) Exp. Cell. Res. 135 221-227.
- Dicker, P. and Rozengurt, E. (1981) Biochem. Biophys. Res. Commun. 100 433-441.
- Doolittle, R.F., Hunkapiller, M.W., Hood, L.E., Devare, S.G., Robbins, K.C., Aaronson, S.A. and Antoniades, H.N. (1983) Science 221 275-277.
- Downes, C.P. and Michell, R.H. (1982) Cell Calcium 3 467-502.
- Downes, C.P. (1986) Trends Neurosci. 11 394-396.
- Downes, C.P., Hawkins, P.T. and Irvine, R.F. (1986) Biochem. J. 238 501-506.
- Downward, J., Yarden, Y., Mayes, E., Scrace, G., Totty, N., Stockwell, P., Ullrich, A., Schlessinger, J. and Waterfield, M.D. (1984) Nature (London) 307 521-527.
- Drummond, A.H. and Raeburn, C.A. (1984) Biochem. J. 224 129-136.
- Drummond, A.H. (1985) Nature (London) 315 752-755.
- Drummond, A.H. (1987) Trends Pharmacol. Rev. 8 129-133.
- Eagle, H., Oyama, V.I., Levy, M. and Freeman, A.E. (1957) J. Biol. Chem. 226 191-205.

- Eigenbrodt, E., Fister, P., Rubsamen, H. and Friis, R.R.
(1983) EMBO J. 2 1565-1570.
- Ek, B., Westermarck, B., Wasteson, Å. and Heldin, C-H. (1982)
Nature (London) 295 419-420.
- Ek, B. and Heldin, C-H. (1984) J. Biol. Chem. 259 11145-11152.
- Ek, B., Rønnstrand, L. and Heldin, C-H. (1984) Biochem. Soc.
Trans. 12 759-762.
- Emilsson, A. and Sundler, R. (1984) J. Biol. Chem. 259
3111-3116.
- Erikson, R.L., Purchino, A.F., Erikson, E., Collett, M.S.
and Brugge, J.S. (1980) J. Cell Biol. 87 319-325.
- Fain, J.N. and Malbon, C.C. (1979) Molec. Cell Biochem. 25
143-148.
- Farese, R.V., Barnes, D.E., Davies, J.S., Standaert, M.L.
and Pollet, R.J. (1984) J. Biol. Chem. 259 7094-7100.
- Ferreri, N.R., Schwartzman, M., Ibrahim, N.G., Chandler, P.N.,
McGiff, J.C. (1984) J. Pharmacol. Exp. Therap. 231
441-448.
- Foulkes, J.G., Cohen, P., Strada, S., Everson, W.V. and
Jefferson, L.S. (1982) J. Biol. Chem. 257 12493-12496.
- Friedman, B., Frackelton, A.R., Ross, A.H., Connors, J.M.,
Fujiki, H., Sugimura, T. and Rosner, M.R. (1984)
Proc. Natl. Acad. Sci. (USA) 81 3034-3038.
- Froesch, E.R., Bürg, H., Ramseier, E.B., Bally, P. and
Labhart, A. (1963) J. Clin. Invest. 42 1816-1834.
- Fujiki, H., Tanaka, Y., Miyake, R., Kikkawa, U., Nishizuka,
Y. and Sugimura, T. (1984) Biochem. Biophys. Res.
Commun. 120 339-343.
- Fürstenberger, G. and Marks, F. (1980) Biochem. Biophys.
Res. Commun. 92 749-756.
- Geisow, M.J., Fritsche, U., Hexham, J.M., Dash, B., and
Johnson, T. (1986) Nature (London) 320 636-638.
- Gill, G.N. and Lazar, C.S. (1981) Nature (London) 293 305-307.
- Girard, D.J., Aaronsen, S.A., Todaro, G.J., Arstein, P.,
Kirsey, J.H., Dosik, H. and Parks, W.P. (1973)
J. Natl. Cancer Inst. 51 1417-1421.

- Glenney, J.R. Jr. (1985) FEBS Lett. 192 79-82.
- Glenney, J.R. Jr. and Tack, B.F. (1985) Proc. Natl. Acad. Sci. (USA) 82 7884-7888.
- Glenney, J.R. Jr. (1986a) J. Biol. Chem. 261 7247-7252.
- Glenney, J.R. Jr. (1986b) Proc. Natl. Acad. Sci. (USA) 83 4258-4262.
- Gomperts, B.D. (1983) Nature (London) 306 64-66.
- Gonzales, R.A. and Crews, F.T. (1985) Biochem. J. 232 799-804.
- Grado, C. and Ballou, C.E. (1961) J. Biol. Chem. 236 54-60.
- Graf, T. and Beug, H. (1978) Biochim. Biophys. Acta 516 269-299.
- Gregory, H. (1975) Nature (London) 257 325-327.
- Greenberg, M.E. and Ziff, E.B. (1983) Nature (London) 311 433-438.
- Habenicht, A.J.R., Glomset, J.A., King, W.C., Nist, C., Mitchell, C.D. and Ross, R. (1981) J. Biol. Chem. 256 12329-12335.
- Habenicht, A.J.R., Goerig, M., Grulich, J., Rothe, D., Gronwald, R., Loth, U., Schettler, G., Kommerell, B. and Ross, R. (1985a) J. Clin. Invest. 75 1381-1387.
- Habenicht, A.J.R., Goerig, M., Grulich, J., Rothe, D., Gronwald, R., Schettler, G. and Konmerell, B. (1985b) Adv. Prost. Thromb. Leukotriene Res. 13 37-39.
- Haigler, H., Ash, J.F., Singer, S.J. and Cohen, S. (1978) Proc. Natl. Acad. Sci. (USA) 75 3317-3321.
- Hallcher, L.M. and Sherman, W.R. (1980) J. Biol. Chem. 225 10896-10901.
- Hamberg, M. and Samuelsson, B. (1974) Proc. Natl. Acad. Sci. (USA) 71 3400-3404.
- Hamberg, M. (1976) Biochim. Biophys. Acta 431 651-654.
- Hammarström, S. (1977) Eur. J. Biochem. 74 7-12.
- Hammarström, S. (1982) Arch. Biochem. Biophys. 214 431-445.
- Hansson, A., Serhan, C.N., Haeggstrom, J., Ingelman-Sundberg, M., Samuelsson, B. and Morris, J. (1986) Biochem. Biophys. Res. Commun. 134 1215-1222.

Hasegawa-Sasaki, H. (1985) *Biochem. J.* 232 99-109.

Hashimoto, Y., Naito, C., Kume, S., Kato, H., Watanabe, T., Kawamura, M., Teramoto, T. and Oka, H. (1985) *Biochim. Biophys. Acta* 841 283-291.

Haslam, R.J. and Davidson, M.M.L. (1984) *FEBS Lett.* 174 90-94.

Hawkins, P.T., Stevens, L. and Downes, C.P. (1986) *Biochem. J.* 238 507-516.

Heikkila, R., Schwab, G., Wickstom, E., Loke, S.L., Pluznik, D.H., Watt, R., Neckers, L.M. *Nature (London)* 328 445-449.

Heldin, C-H, Westermarck, B. and Wasteson, Å. (1979) *Proc. Natl. Acad. Sci. (USA)* 76 3722-3726.

Heldin, C-H., Ek, B. and Rønnstrand, L. (1983) *J. Biol. Chem.* 258 10054-10061.

Herschman, H.R., Sorrentino, J.M., Butler-Gralla, E.R., Crawley, J.B. (1982) in *Progress in Cancer Res. and Therapy.* (Moore, M.A.S. ed.) 23 41-45 (Raven Press, New York).

Hesketh, T.R., Moore, J.P., Morris, J.H.M., Taylor, M.V., Rogers, J., Smith, G.A. and Metcalfe, J.C. (1985) *Nature (London)* 313 481-484.

Heslop, J.P., Blakeley, D.M., Brown, K.D., Irvine, R.F., Berridge, M.J. (1986) *Cell* 47 703-709.

Higgs, G.A., Flower, R.J. and Vane, J.R. (1979) *Biochem. Pharmacol.* 28 159-161.

Hirata, F., Schiffman, E., Venkatasubramanian, K., Salomon, D. and Axelrod, J. (1980) *Proc. Natl. Acad. Sci. (USA)* 77 2533-2536.

Hirata, F., Notsu, Y., Iwata, M., Parente, L., Dirosa, M. and Flower, R.J. (1982) *Biochem. Biophys. Res. Commun.* 109 223-230.

Hirata, F. (1983) *Adv. Prost. Thromb. Leuko. Res.* 11 73-78.

Hoak, J.C., Warner, E.D. and Connor, W.E. (1967) *Circul. Res.* 20 11-17.

- Hokin, M.R. and Hokin, L.E. (1953) J. Biol. Chem. 203 967-977.
- Hokin, L.E. (1985) Ann. Rev. Biochem. 54 205-235.
- Holley, R.W. and Kiernan, J.A. (1968) Proc. Natl. Acad. Sci. (USA) 60 300-304.
- Holley, R.W. (1975) Nature (London) 258 487-490.
- Hong, S.L. and Deykin, D. (1981) J. Biol. Chem. 256 5215-5219.
- Houslay, M.D. (1985) in Molecular Mechanisms of Transmembrane Signalling (Cohen, S. and Houslay, M.D. eds.) 279-334 (Elsevier Science).
- Houslay, M.D., Bojanic, D. and Wilson, A. (1986) Biochem. J. 234 737-740.
- Huang, K-S., Wallner, B.P., Mattaliano, R.J., Tizard, R., Bourne, C., Frey, A., Hession, C., McGray, P., Sinclair, L., Chow, E.P., Browning, J., Ramachandran, K., Tang, J., Smart, J. and Pepinsky, R.B. (1986) Cell 46 191-199.
- Hughes, P.J. and Drummond, A.H. (1987) Biochem. J. 248 463-470.
- Hunter, T. and Setton, B.M. (1980) Proc. Natl. Acad. Sci. (USA) 77 1311-1315.
- Hunter, T., Ling, N. and Cooper, J.A. (1984) Nature (London) 311 480-483.
- Hunter, T. and Cooper, J.A. (1985) Ann. Rev. Biochem. 54 897-930.
- Hutton, J.C. (1986) Cell Calcium 7 339-352.
- Imai, A. and Gershengorn, M.C. (1986) Proc. Natl. Acad. Sci. (USA) 83 8540-8544.
- Irvine, R.F. (1982) Biochem. J. 204 3-16.
- Irvine, R.F., Letcher, A.J., Lander, D.J. and Downes, C.P. (1984) Biochem. J. 223 237-243.
- Irvine, R.F., Letcher, A.J., Heslop, J.P. and Berridge, M.J. (1986) Nature (London) 320 631-634.
- Irvine, R.F. and Moor, R.M. (1986) Biochem. J. 260 917-920.

- Jacob, S. and Cuatrecasas, P. (1981) *Endocrinol. Rev.* 2 251-263.
- Jacob, S., Kull, F.C. Jr., Earp, H.S., Suoboda, M.E., Van Wyk, J.J. and Cuatrecasas, P. (1983) *J. Biol. Chem.* 258 9581-9504.
- Jetten, A.M., Ganong, B.R., Vandenbark, G.R., Shirley, J.E. and Bell, R.M. (1985) *Proc. Natl. Acad. Sci. (USA)* 82 1941-1945.
- Jimenez de Asua, L., Clingan, L.D., Rudland, P.S. (1975) *Proc. Natl. Acad. Sci. (USA)* 73 2724-2728.
- Jimenez de Asua, L., Richmond, K.M.V., O'Farrell, M.K., Otto, A.M., Kubler, A.M. and Rudland, P.S. (1979) in *Hormones and Cell Culture: Cold Spring Harbour Conferences in Cell Proliferation* (Sato, G.H. and Ross, R. eds.) 6 404-424 (Cold Spring Harbour Lab.: Cold Spring Harbour, New York).
- Jimenez de Asua, L., Richmond, K.M.V. and Otto, A.M. (1981) *Proc. Natl. Acad. Sci. (USA)* 78 1004-1008.
- Jimenez de Asua, L., Otto, A.M., Lindgren, J.A. and Hammarström, S. (1983) *J. Biol. Chem.* 259 8774-8780.
- Johnsson, A., Heldin, C-H., Westermark, B. and Wasteson, Å. (1982) *Biochem. Biophys. Res. Commun.* 104 66-74.
- Johnsson, A., Heldin, C-H., Wasteson, Å., Westermark, B., Deuel, T.F., Huang, J.S., Seeburg, D.H., Gray, E., Ullrich, A., Scrace, G., Stroobant, P. and Waterfield, M.D. (1984) *EMBO J.* 3 921-928.
- Johnsson, A., Betsholtz, C., Helm, K. von der., Heldin, C-H., and Westermark, B. (1985) *Proc. Natl. Acad. Sci. (USA)* 82 1721-1725.
- Jolles, J., Zwiess, H., Dekker, A., Wirtz, K.W.A. and Gispen, W.H. (1981) *Biochem. J.* 194 283-291.
- Kaibuchi, K., Takai, U., Nishizuka, U. (1985) *J. Biol. Chem.* 260 1366-1369.
- Kasuga, M., Van Obberghen, E., Nissley, S.P. and Rechler, M.M. (1981) *J. Biol. Chem.* 256 5305-5308.
- Kasuga, M., Zick, Y., Blithe, D.L., Karlsson, F.A., Håring, H.U. and Kahn, C.R. (1982a) *J. Biol. Chem.* 257 9891-9894.

- Kasuga, M., Zick, Y., Blithe, D.L., Crettaz, M., Kahn, C.R.
(1982b) *Nature (London)* 298 667-669.
- Kato, N., Halprin, K.M., Matsuo, S., Taylor, J.R. (1985)
Biochem. Biophys. Res. Commun. 130 761-760.
- Katz, D.H. (1977) in *Lymphocyte Differentiation, Recognition and Regulation*, 247-342 (Academic Press, New York).
- Kelly, K., Cochran, B.H., Stiles, C.D. and Leder, P. (1983)
Cell 35 603-610.
- Kikkawa, U., Takai, Y., Minakuchi, P., Inoh, J.S. and Nishizuka, Y. (1982) *J. Biol. Chem.* 257 13341-13348.
- King, A.C. and Cuatrecasas, P. (1982) *J. Biol. Chem.* 257 3053-3060.
- Kingston, R.E., Baldwin, A.S. and Sharp, P.A. (1985) *Cell* 41 3-5.
- Kohn, M. (1985) *J. Biol. Chem.* 260 1771-1779.
- Kolesnick, R.N. and Gershengorn, M.C. (1985) *J. Biol. Chem.* 260 707-713.
- Kruijer, W., Cooper, J.A., Hunter, T. and Verma, I.M. (1984)
Nature (London) 312 711-716.
- Kruszynski, L.B., Manning, M., Seto, J., Haldar, J., Sawyer, W.H. (1980) *J. Med. Chem.* 23 364-368.
- Kuksis, A. and Mookerjee, M. (1978) *Nutrition Rev.* 36 233-238.
- Kuo, J.F., Anderson, R.G.G., Wise, R.C., Mackerlova, L., Salmonsson, I., Brackett, N.L., Katoh, N., Shoji, M. and Wrenn, R.W. (1980) *Proc. Natl. Acad. Sci. (USA)* 77 7039-7043.
- Lanks, K.W., Kasambalides, E.J., Chinkers, M. and Brugge, J.S. (1982) *J. Biol. Chem.* 257 8604-8607.
- Lee, L.S. and Weinstein, I.B. (1978) *Nature (London)* 274 696-697.
- Leeb-Lundberg, F.L.M., Cotecchia, S., Lomasney, J.W., De Bernardis, J.F., Lefkowitz, R.L. and Caron, M.J. (1985) *Proc. Natl. Acad. Sci. (USA)* 82 5651-5655.
- Levine, L. (1982) in *Prostaglandins and Cancer: Prost. and Related Lipids* (Powles, T.J., Bockman, R.S., Honn, K.V. and Ranwell, P., eds.) 189-204 (Alan R. Liss Inc., New York).

- Londos, C., Cooper, D.M.F. and Wolff, J. (1980) Proc. Natl. Acad. Sci. (USA) 77 2551-2554.
- Lopez-Rivas, A. and Rozengurt, E. (1984) Amer. J. Physiol. (Cell Physiol.) 247 C156-C162.
- Lowry, O.H., Rosenbrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193 256-275.
- Martin, R.G. (1981) Adv. Cancer Res. 34 1-68.
- Massagué, J., Pilch, P.F. and Czech, M.P. (1980) Proc. Natl. Acad. Sci. (USA) 77 7137-7141.
- Massagué, J. and Czech, M.P. (1982) J. Biol. Chem. 257 5038-5045.
- Mastro, A.M. and Müeller, G.C. (1974) Exp. Cell Res. 88 40-46.
- Mellstrom, K., Höglund, A-S., Nister, M., Heldin, C-H., Westermarck, B. and Linberg, U. (1983) J. Muscle Res. Cell Motil. 4 589-609.
- Mendoza, S.A., Wigglesworth, N.M. and Rozengurt, E. (1980) J. Cell Physiol. 105 153-162.
- Mendoza, S.A., Schneider, J.A., Lopez-Rivas, A., Sinnett-Smith, J.W. and Rozengurt, E. (1986) J. Cell Biol. 102 2223-2233.
- Michell, R.H. (1975) Biochim. Biophys. Acta 415 81-147.
- Michell, R.H. (1979) Trends Biochem. Sci. 4 128-131.
- Michell, R.H., Kirk, C.J., Jones, L.M., Downes, C.P. and Creba, J.A. (1981) Philos. Trans. R. Soc. London (Ser. V) 296 123-137.
- Michell, R.H. (1982) Cell Calcium 3 429-440.
- Milligan, G. and Klee, W.A. (1985) J. Biol. Chem. 260 2057-2063.
- Minamino, N., Kangawa, K. and Matsuo, H. (1983) Biochem. Biophys. Res. Commun. 114 541-548.
- Minamino, N., Kangawa, K. and Matsuo, H. (1984) Biochem. Biophys. Res. Commun. 119 14-20.
- Minna, J.D. and Gilman, A.G. (1973) J. Biol. Chem. 248 6613-6625.

- Mix, L.L., Dinerstein, R.J. and Villereal, M.L. (1984) Biochem. Biophys. Res. Commun. 119 69-75.
- Moolenaar, W.H., Tsien, R.Y., Van der Saag, P.T. and de Laat, S.W. (1983) Nature (London) 304 645-648.
- Moolenaar, W.H., Tertoolen, L.G.J. and de Laat, S.W. (1984a) Nature (London) 312 371-374.
- Moolenaar, W.H., Tertoolen, L.G.J. and de Laat, S.W. (1984b) J. Biol. Chem. 259 8066-8069.
- Morgan, D.O., Edman, J.C., Standring, D.N., Fried, V.A., Smith, M.C., Roth, R.A. and Rutter, W.J. (1987) Nature (London) 329 301-307.
- Morris, J.D.H., Metcalfe, J.C., Smith, G.A., Hesketh, T.R., and Taylor, M.V. (1984) FEBS Lett. 169 189-193.
- Morrison, A.R. and Pascoe, N. (1981) Proc. Natl. Acad. Sci. (USA) 78 7375-7378.
- Müller, R., Bravo, R., Burckhardt, J. and Curran, T. (1984) Nature (London) 312 716-720.
- Murayama, T. and Ui, M. (1985) J. Biol. Chem. 260 7226-7233.
- Macphee, C.H. and Drummond, A.H. (1984) Mol. Pharmacol. 25 193-200.
- Macphee, C.H., Drummond, A.H., Otto, A.M. and Jimenez de Asua, L. (1984) J. Cell Physiol. 119 35-40.
- McDonald, T.J., Jornvall, H., Nilsson, M., Vagne, M., Ghatei, M., Bloom, S.R. and Mutt, V. (1979) Biochem. Biophys. Res. Commun. 90 227-233.
- McPhail, L.C., Clayton, C.C. and Snyderman, R. (1984) Science 224 622-624.
- Nakamura, K.D., Martinez, R. and Weber, M.J. (1983) Molec. Cell Biol. 3 380-390.
- Needleman, P., Turk, J., Jakschik, B.A., Morrison, A.R. and Lefkowitz, J.B. (1986) Ann. Rev. Biochem. 55 69-102.
- Nigg, E.A., Cooper, J.A. and Hunter, T. (1983) J. Cell Biol. 96 1601-1609.
- Nishimura, J., Huang, J.S., Deuel, T.F. (1982) Proc. Natl. Acad. Sci. (USA) 79 4303-4307.

- Nishizuka, Y. (1983) Trends Biochem. Sci. 8 13-16.
- Nishizuka, Y. (1984) Science 225 1365-1370.
- Nishizuka, Y. (1986) Science 233 305-312.
- Northrup, J.K. (1985) in Molecular Mechanisms of Transmembrane Signalling (Cohen, S. and Houslay, M.D. eds.) 91-129 (Elsevier Science).
- O'Brian, C.A., Arcoleo, J.P., Housey, G.M. and Weinstein, I.B. (1985) in Cancer Cells (Feramisco, J., Ozanne, B. and Stiles, C.D. eds.) 359-364. (Cold Spring Harbour, New York).
- Ochoa, S. (1985) Prog. Med. Virol. 32 1-4.
- O'Farrell, M.K., Clingan, D., Rudland, P.S., Jimenez de Asua, L. (1979) Exp. Cell Res. 118 311-312.
- Oliw, E.H., Lawson, J.A., Brash, A.R. and Oates, J.A. (1981) J. Biol. Chem. 256 9924-9931.
- Oppermann, H., Levinson, A.D., Levintow, L., Varmus, H.E., Bishop, J.M. and Kawai, S. (1981a) Virology 113 736-751.
- Oppermann, H., Levinson, A.D. and Bishop, J.M. (1981b) Proc. Natl. Acad. Sci. (USA) 78 1067-1071.
- Otto, A.M., Nilsen-Hamilton, M., Boss, B.D., Ulrich, M.O. and Jimenez de Asua, L. (1982) Proc. Natl. Acad. Sci. (USA) 79 4992-4996.
- Pace-Asciak, C.R. and Smith, W.L. (1983) Enzymes 16 543-603.
- Parker, P.J., Coussens, L., Totty, N., Rhee, L., Young, S., Chen, E., Stabel, S., Waterfield, M.D. and Ullrich, A. (1986) Science 233 853-859.
- Pepinsky, R.B. and Sinclair, L.K. (1986) Nature (London) 321 81-84.
- Pessin, J.E., Gitomer, W., Oka, Y., Oppenheimer, C.L. and Czech, M.P. (1983) J. Biol. Chem. 258 7386-7394.
- Pledger, W.J., Stiles, C.D., Antoniades, H.N. and Scher, C.D. (1977) Proc. Natl. Acad. Sci. (USA) 74 4481-4485.
- Poffenbarger, P.L. (1968) J. Clin. Invest. 47 2678-2688.

- Pollock, W.K., Rink, T.J. and Irvine, R.F. (1986) *Biochem. J.* 235 869-877.
- Powles, T.J., Bockman, R.S., Honn, K.V. and Ranwell, P. (1982) in *Prostaglandins and Cancer: Prost. and Related Lipids* (Alan R. Liss Inc., New York).
- Putney, J.W. Jr. (1978) *Pharmacol. Rev.* 30 209-245.
- Putney, J.W. Jr. (1981) *Life Sci.* 29 1183-1194.
- Putney, J.W. Jr., Poggioli, J. and Weiss, S.J. (1981) *Philos. Trans. R. Soc. London (B. Biol. Sci.)* 296 37-45.
- Putney, J.W. Jr., Aub, D.L., Taylor, C.W. and Merritt, J.E. *Fedn. Proc.* 45 2634-2638.
- Rabin, M.S., Doherty, P.J. and Gottesman, M.M. (1986) *Proc. Natl. Acad. Sci. (USA)* 83 357-360.
- Radke, K. and Martin, G.S. (1979) *Proc. Natl. Acad. Sci. (USA)* 76 5212-5216.
- Radke, K., Carter, C., Moss, P., Dehazya, P., Schliwa, M. and Martin, G.S. (1983) *J. Cell Biol.* 97 1601-1611.
- Ramstedt, U., Ng, J., Wigzell, H., Serhan, C.N. and Samuelsson, B. (1985) *J. Immunology* 135 3434-3438.
- Riedel, H., Dull, T.J., Schlessinger, J. and Ullrich, A. (1986) *Nature (London)* 324 68-70.
- Rinderknecht, E. and Humbel, R.E. (1978a) *J. Biol. Chem.* 253 2769-2776.
- Rinderknecht, E. and Humbel, R.E. (1978b) *FEBS Lett.* 89 283-286.
- Ristow, H.J., Messmer, T.O., Walter, S. and Paul, D. (1980) *J. Cell Physiol.* 103 263-269.
- Rittenhouse-Simmons, S. (1981) *J. Biol. Chem.* 256 4153-4155.
- Rittenhouse, S.E. and Sasson, J.P. (1985) *J. Biol. Chem.* 260 8657-8661.
- Robbins, K.C., Antoniades, H.N., Devare, S.G., Hunkapiller, M.W. and Aaronson, S.A. (1983) *Nature (London)* 305 605-608.
- Rodbell, M. (1980) *Nature (London)* 284 17-22.
- Rodriguez-Pena, A. and Rozengurt, E. (1986) *J. Cell Physiol.* 129 124-130.

- Rosoff, P.M., Stein, L.F. and Cantley, L.C. (1984) J. Biol. Chem. 259 7056-7060.
- Rosner, M.R., McCaffrey, P.G., Friedman, B. and Foulkes, J.G. (1985) In Cancer Cells (Feramisco, J., Ozanne, B. and Stiles, C.D. eds.) 347-351 (Cold Spring Harbour Lab., New York).
- Rothhut, B., Russo-Marie, F., Wood, J., Dirosa, M. and Flower, R.J. (1983) Biochem. Biophys. Res. Commun. 117 878-884.
- Rozengurt, E., Legg, A., and Pettican, P. (1979) Proc. Natl. Acad. Sci. (USA) 76 1284-1287.
- Rozengurt, E. (1980) Curr. Topics Cell Reg. 17 59-88.
- Rozengurt, E., Brown, K.D. and Pettican, P. (1981a) J. Biol. Chem. 256 715-722.
- Rozengurt, E., Legg, A., Strang, G., Courtney-Luck, N. (1981b) Proc. Natl. Acad. Sci. (USA) 78 4392-4396.
- Rozengurt, E., Gelehrter, T.D., Legg, A. and Pettican, P. (1981c) Cell 23 781-788.
- Rozengurt, E. (1982a) J. Cell Physiol. 112 243-250.
- Rozengurt, E. (1982b) Exp. Cell Res. 139 71-78.
- Rozengurt, E. and Sinnett-Smith, J.W. (1983) Proc. Natl. Acad. Sci. (USA) 80 2936-2940.
- Rozengurt, E., Rodriguez-Pena, M. and Smith, K.A. (1983a) Proc. Natl. Acad. Sci. (USA) 80 7244-7248.
- Rozengurt, E., Collins, M.K.L. and Keehan, M. (1983b) J. Cell Physiol. 116 379-384.
- Rozengurt, E., Stroobant, P., Waterfield, M.D., Deuel, T.F. and Keehan, M. (1983c) Cell 34 265-272.
- Rozengurt, E., Rodriguez-Pena, A., Coombs, M., Sinnett-Smith, J.W. (1984) Proc. Natl. Acad. Sci. (USA) 81 5748-5752.
- Rozengurt, E. and Sinnett-Smith, J.W. (1987) J. Cell Physiol. 131 218-225.
- Rozengurt, E., Murray, M., Zachary, I., Collins, M. (1987) Proc. Natl. Acad. Sci. (USA) 84 2282-2286.
- Rubin, J.B., Shia, M.A., Pilch, P.F. (1983) Nature (London) 305 438-440.

- Salmon, J.A. (1986) Adv. Drug Res. 15 111-167.
- Samuelsson, B., Goldyne, M., Grastrom, E., Hamberg, M., Hammarström, S. and Malmstein, C. (1978) Ann. Rev. Biochem. 47 997-1029.
- Samuelsson, B. (1983) Science 220 568-575.
- Saris, C.J.M., Kristensen, T., Tack, B.F., Glenney, J.R. Jr. and Hunter, T. (1986) Cell 46 202-212.
- Savage, C.R. Jr. and Cohen, S. (1972) J. Biol. Chem. 247 7609-7611.
- Savage, C.R. Jr., Inagami, T. and Cohen, S. (1972) J. Biol. Chem. 247 7612-7621.
- Savage, C.R. Jr., Hash, J.H. and Cohen, S. (1973) J. Biol. Chem. 248 7669-7672.
- Sawyer, S.T. and Cohen, S. (1981) Biochem. J. 20 6280-6286.
- Schacht, J. (1981) Methods in Enzymology 72 623-631.
- Schuldiner, S. and Rozengurt, E. (1982) Proc. Natl. Acad. Sci. (USA) 79 7778-7782.
- Serhan, C.N., Hamberg, M. and Samuelsson, B. (1984a) Biochem. Biophys. Res. Commun. 118 943-949.
- Serhan, C.N., Hamberg, M. and Samuelsson, B. (1984b) Proc. Natl. Acad. Sci. (USA) 81 5335-5339.
- Serhan, C.N., Hamberg, M., Ramstedt, U. and Samuelsson, B. (1986) Adv. Prost. Thromb. Leuko. Res. 16 83-97.
- Sherman, W.R., Gish, B.G., Honchar, M.P. and Munsell, L.Y. (1986) Fedn. Proc. 45 2639-2646.
- Shier, W.T. (1979) Proc. Natl. Acad. Sci. (USA) 76 195-199.
- Shier, W.T. (1980) in Natural Toxins (Eaker, D. and Wadstrom, T. eds.) 193-200 (Pergamon Press, Oxford and New York).
- Shier, W.T. and Trotter, J.T. (1980) Biochim. Biophys. Acta 619 235-246.
- Shier, W.T. and Durkin, J.P. (1982) J. Cell Physiol. 112 171-181.
- Shoam, H. and Razin, E. (1985) Biochim. Biophys. Acta 837 1-5.

- Shoyab, M., De Larco, J.E. and Todaro, G.J. (1979) *Nature* (London) 279 387-391.
- Siess, W., Cuatrecasas, P. and Lapetina, E.G. (1983) *J. Biol. Chem.* 258 4683-4686.
- Slepian, I.K., Mathews, K.P. and McLean, J.A. (1985) *Chest* 87 386-391.
- Smigel, M., Katada, T., Northrup, J.K., Bokoch, G.M., Ui, M. and Gilman, A.G. (1984) *Adv. Cycl. Nucl. Prot. Phosphoryl. Res.* 17 1-18.
- Sreevalsan, T., Rozengurt, E., Taylor-Papadimitriou, J. and Burchell, J. (1980) *J. Cell Physiol.* 104 1-9.
- Streb, H., Irvine, R.F., Berridge, M.J. and Schultz, I. (1983) *Nature* (London) 306 67-68.
- Streb, H., Bayerdörffer, E., Haase, W., Irvine, R.F. and Schultz, I. (1984) *J. Mem. Biol.* 81 241-253.
- Stroobant, P., Gullick, W.J., Waterfield, M.D. and Rozengurt, E. (1985) *EMBO J.* 4 1945-1949.
- Struani, E., Vincentini, L.M., Zippel, R., Toschi, L., Pandiella-Alonso, A., Comoglio, P.M. and Meldolesi, J. (1986) *Biochem. Biophys. Res. Commun.* 137 343-350.
- Takai, Y., Kishimoto, A., Iwasa, U., Kawahara, Y., Mori, T. and Nishizuka, Y. (1979) *J. Biol. Chem.* 254 3692-3695.
- Takayama, S., White, M., Lauris, V. and Kahn, C.R. (1984) *Proc. Natl. Acad. Sci. (USA)* 81 7797-7801.
- Taylor, J.M., Michell, W.M. and Cohen, S. (1972) *J. Biol. Chem.* 247 5928-5934.
- Taylor, L. and Polgar, P. (1977) *FEBS Lett.* 79 69-72.
- Todaro, G.J. and Green, H. (1963) *J. Cell Biol.* 17 299-313.
- Touqui, L., Rothhut, B., Shaw, A.M., Fradin, A., Vargaftig, B.B. and Russo-Marie, F. (1986) *Nature* (London) 321 177-180.
- Turk, J., Maas, R., Brash, A.R., Jackson-Roberts, L. II and Oates, J.A. (1982) *J. Biol. Chem.* 257 7068-7076.
- Ubatuba, F.B. (1978) *J. Chromatog.* 161 165-177.

- Ushiro, H. and Cohen, S. (1981) Nature (London) 290
516-519.
- Vincentini, L.M., Miller, R.J. and Villereal, M.L. (1984)
J. Biol. Chem. 259 6912-6919.
- Volpi, M., Yassin, R., Tao, W., Molski, T.F.P., Naccach,
P.H. and Sha'afi, R.I. (1981) Proc. Natl. Acad. Sci.
(USA) 81 5966-5969.
- Wakelam, M.J.O., Davies, S.A., Houslay, M.D., McKay, I.,
Marshall, C.J. and Hall, A. (1986) Nature (London)
323 173-176.
- Wallner, B.P., Mattaliano, R.J., Hession, C., Cate, R.L.,
Tizard, R., Sinclair, L.K., Foeller, C., Chow, E.P.,
Browning, J.L., Ramachandran, K. and Pepinsky, R.B.
(1986) Nature (London) 320 77-81.
- Waterfield, M.D., Scrace, T., Whittle, N., Stroobant, P.,
Johnsson, A., Wasteson, Å., Westermarck, B., Heldin,
C-H., Huang, J-S. and Deuel, T.F. (1983) Nature (London)
304 35-39.
- Wickstrom, E.L., Wickstrom, E., Lyman, G.H. and Freeman, D.L.
(1986) Fedn. Proc. 45 1708-1712.
- Wolf, B.A., Turk, J., Sherman, W.R. and Daniel, M.L. (1986)
J. Biol. Chem. 261 3501-3511.
- Wong, P. Y-K., Hughes, R. and Lam, B. (1985) Biochem.
Biophys. Res. Commun. 126 763-772.
- Yarden, Y., Escobedo, J.A., Kuang, W.-J., Yang-Feng, T.L.,
Daniel, T.O., Tremble, P.M., Chen, E.Y., Ando, M.E.,
Harkins, R.N., Francke, V., Fried, V.A., Ullrich, A.
and Williams, L.T. (1986) Nature (London) 323 226-232.
- Yavin, E. and Zutra, A. (1977) Analyt. Biochem. 80 430-437.
- Yoshimasa, T., Sibley, D.R., Bouvier, M., Lefkowitz, R.J.
and Caron, M.G. (1987) Nature (London) 327 67-70.
- Zachary, I. and Rozengurt, E. (1985) Proc. Natl. Acad. Sci.
(USA) 82 7616-7620.
- Zachary, I. and Rozengurt, E. (1987) J. Biol. Chem. 262
3947-3950.
- Zapf, J., Froesch, E.R. and Humbel, R.E. (1981) Curr. Topics
Cell Reg. 19 257-309.
- Zeitler, P. and Handwerker, S. (1985) Mol. Pharmacol. 28
549-554.